Antimicrobial resistance patterns of clinical and non-clinical enterococcal isolates at Kiambu District Hospital.

By

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Reg. No. H56/P/7284/2004

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL MICROBIOLOGY AT THE UNIVERSITY OF NAIROBI
DEDICATION

I dedicate this project to my late grandmother Sermetei Kokoyo who first took me to school, and to my son Meitamei Oseur Naikuni.
DECLARATION

This is my own original work and has not been presented for a degree in any other university to the best of my knowledge.

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ACKNOWLEDGMENTS

I greatly appreciate the encouragement, patience and guidance of my supervisors Prof. Walter Jaoko and Prof. Gunturu Revathi.

I also acknowledge all those who assisted me while joining the masters degree program including my parents and all my brothers and extended family. May the good lord bless you all.

Special acknowledgment goes to my brother in-law Lenku Kanar and Sister Ruth Nasinkoi Lenku who were my guardian angels in this program.

The Lord will abundantly bless you.
# ABREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AREF</td>
<td>Ampicillin resistant <em>Enterococcus faecium</em></td>
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<tr>
<td>AS</td>
<td>Aggregation substance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standards Institute.</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBS</td>
<td>Enterococcal binding substance</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>HL</td>
<td>High level</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive Care Unit</td>
</tr>
<tr>
<td>IE</td>
<td>Infective endocarditis</td>
</tr>
<tr>
<td>LD</td>
<td>Lethal Dose 50</td>
</tr>
<tr>
<td>LLC-PIC</td>
<td>Cultured pig renal tubular cell line</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>MBC</td>
<td>Minimum bactericidal concentration</td>
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<tr>
<td>MDR</td>
<td>Multiple-drug resistant</td>
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<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin resistant staphylococcus aureus</td>
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<tr>
<td>NNIS</td>
<td>National Nosocomial Infections Surveillance</td>
</tr>
<tr>
<td>PAD</td>
<td>Pheromone responsive plasmid</td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin binding proteins</td>
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<tr>
<td>PMN</td>
<td>Polymorphonuclear cells</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
</tr>
<tr>
<td>Van A</td>
<td>Resistance to vancomycin and teicoplanin</td>
</tr>
<tr>
<td>Van B</td>
<td>Resistance to vancomycin alone</td>
</tr>
<tr>
<td>Van C</td>
<td>Low level resistance to vancomycin</td>
</tr>
<tr>
<td>VRE</td>
<td>Vancomycin resistant enterococci</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>i</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENT</td>
<td>iii</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
</tbody>
</table>

## 1.0 CHAPTER I - INTRODUCTION

1.1 Objectives of the study 2

1.2 Problem statement and justification 2

## 2.0 CHAPTER II - LITERATURE REVIEW

2.1 Habitat, microbiology, and epidemiology 4

2.2 Pathogenic mechanisms 7

2.2.1 Colonization and translocation 7

2.2.2 Bactereamia 9

2.2.3 Urinary tract infection 9

2.2.4 Endocarditis 10

2.2.5 Endopthalmitis 10

2.2.6 Immune evasion 11

2.2.7 Pathologic tissue damage 12

2.2.8 Indirect tissue damage 12

2.2.9 Direct tissue damage 13

2.3 Antimicrobial resistance 14

2.4 Mechanisms of resistance 16
2.4.1 β-lactam resistance 16

2.4.2 Aminoglycoside resistance 17

2.4.3 Vancomycin resistance 17
  2.4.3.1 VanA glycopeptide resistance 18
  2.4.3.2 VanB glycopeptide resistance 19
  2.4.3.3 VanC glycopeptide resistance 20
  2.4.3.4 VanD glycopeptide resistance 20
  2.4.3.5 VanE glycopeptide resistance 21
  2.4.3.6 Vancomycin-dependent enterococci 21

2.5 Therapeutic approaches 21

2.6 Infection control 24

3.0 CHAPTER III - MATERIALS AND METHODS 26

3.1 Study design 26

3.2 Study population 26

3.3 Patient data 26

3.4 Study area 26

3.5 Sampling method 26

3.6 Inclusion/exclusion criteria 26

3.7 Sample size calculation 26

3.8 Study procedures 27

3.9 Recruitment procedure for healthy controls 27

3.10 Isolates 27

3.11 Identification 27

3.12 Tests 27
LIST OF TABLES

Table 1.  Distribution of sexes in the study and control groups
Table 2.  Overall distribution of enterococcal species in both clinical and non-clinical samples
Table 3.  Distribution of enterococcal species in clinical samples
Table 4.  Distribution of enterococcal species by specimen
Table 5.  Antimicrobial resistance profile among clinical enterococcal isolates (n=39)
Table 6.  Antimicrobial resistance profile among enterococcal isolates from patient stools (n=50)
Table 7.  Antimicrobial resistance profile among enterococcal isolates from healthy control stools (n=50)
Table 8.  Statistical difference in the frequency of drug resistance between clinical and non-clinical enterococcal isolates.
ABSTRACT
This prospective study was carried out to determine the species distribution and antimicrobial susceptibilities of enterococci isolated from clinical and nonclinical samples at Kiambu District Hospital. Enterococcus species isolated from blood, urine, pus, ascitic fluid and stool specimen between March 2011 and September 2011 were identified by standard biochemical tests. Antimicrobial susceptibility testing was performed by disk diffusion method as per CLSI guidelines. Out of a total of 139 Enterococcus species recovered during the study period, E. faecalis (66, 47.5%) and E. faecium (39, 28%) constituted the predominant isolates (75.5%). There were 34 (24.5%) other enterococcal species isolated. Of the 139 isolates 2 were recovered from blood, 3 from ascitic fluid, 13 from urine, 21 from pus, 50 from patients’ stools and 50 from healthy control’s stools. Among the 39 non-stool clinical isolates, E. faecalis constituted 82%, E. faecium 15.4%. Species other than E. faecalis and E. faecium accounted for 2.6% of the clinical, and for 33% of the faecal isolates. E. faecalis was predominantly isolated from pus, urine, blood and ascitic samples. E. faecium and other enterococcal species were mainly isolated from stool. Distribution of species among patients’ stools and healthy control’s stools were not markedly different. Resistance to vancomycin was not detected. Overall, antibiotic resistance of E. faecium isolates was more than that of E. faecalis or other enterococcal species. Resistance to antibiotics among clinical isolates was higher than that in stool isolates. Resistance to ampicillin was only observed among clinical isolates. Among clinical strains, overall resistance to ampicillin was 7.7%, to gentamycin 17.9 %, and to streptomycin 35.9%. Prevalence of a wide variety of Enterococcus species in clinical samples together with their variable antimicrobial susceptibility patterns emphasizes the need for routinely carrying out detailed speciation and in vitro susceptibility testing of enterococcal isolates in clinical bacteriology laboratories.
CHAPTER I

INTRODUCTION

Enterococci are associated with both community- and hospital-acquired infections. They present a therapeutic challenge because of their resistance to a vast array of antimicrobial drugs, including cell-wall active agents, all commercially available aminoglycosides, penicillin, ampicillin, and vancomycin (Murray 1998). Enterococci exhibit a tendency to acquire resistance. This tendency may relate to their ability to form variations of conjugation, which can result in the spread of genes as part of conjugative transposons, pheromone-responsive plasmids, or broad host-range plasmids. Enterococcal hardiness likely adds to resistance by facilitating survival in the environment of a multidrug-resistant clone, thus enhancing potential spread from person to person. The combination of these attributes within the genus *Enterococcus* suggests that these bacteria and their resistance to antimicrobial drugs will continue to pose a challenge.

The enterococci are now receiving increased attention because of their resistance to multiple antimicrobial drugs, which probably explains a large part of their prominence in nosocomial infections. The most common enterococci-associated nosocomial infections are of the urinary tract, followed by those of surgical wounds, and bacteremia (Moellering, 1992). Enterococci are often present in intra-abdominal and pelvic infections, although not all patients with such infections require specific anti-enterococcal therapy. Other enterococcal infections include meningitis and bacteremia in very ill neonates; central nervous system infections in adults, typically with a history of central nervous system surgery or intrathecal chemotherapy; and rarely, osteomyelitis and pulmonary infections. Enterococci frequently arise from colonization of indwelling tubes, causing liver or biliary tract infection in liver transplant patients (Murray, 1990).

Most enterococcal infections are caused by *Enterococcus faecalis*, which are more likely to express traits related to overt virulence but also more likely to retain sensitivity to at least one effective antibiotic. The remaining infections are mostly caused by *E. faecium*, a species virtually devoid of known overt pathogenic traits but more likely to be resistant to even antibiotics of last resort (Huycke *et al*, 1998). The therapeutic challenge of multiple-drug resistant (MDR)
enterococci - those strains with significant resistance to two or more antibiotics, often including, but not limited to vancomycin -has brought their role as important nosocomial pathogens into sharper focus.

The present study sought to establish the antimicrobial susceptibility patterns of enterococci among clinical and non-clinical enterococcal isolates. It aimed to provide knowledge on the current status of antimicrobial resistance and/or susceptibility of enterococci. It also aimed at providing information on the existence of multi-drug resistant enterococci among those isolates causing clinical infections.

1.1 OBJECTIVES OF THE STUDY

General objective

To determine the antibiotic resistance patterns of enterococci isolated from clinical and non-clinical samples at Kiambu District Hospital.

Specific objectives

1. To determine the antibiotic susceptibility patterns of the enterococcal isolates.
2. To determine the distribution of enterococcal species in both clinical and non-clinical specimen.
3. To identify the probable antibiotic alternatives in enterococcal infection therapy.

1.2 PROBLEM STATEMENT AND JUSTIFICATION

Enterococci have been known to be a cause of infective endocarditis for close to a century. More recently they have been recognized as a cause of nosocomial infection and "superinfection" in patients receiving antimicrobial agents (Murray, 1990). Nosocomial infection and transmission of enterococci is an increasingly prevalent phenomenon. The most common enterococci-associated nosocomial infections are infections of the urinary tract, followed by surgical wound infections and bacteremia (Moellering, 1992).

Two types of enterococci cause infections. The first type are those originating from patients’
native flora, which are unlikely to possess resistance beyond that intrinsic to the genus, and are unlikely to be spread from bed to bed. The second types are isolates that possess multiple antibiotic resistance traits and are capable of nosocomial transmission.

The hospital setting is a high-risk area for enterococcal infection mainly because of two reasons. Firstly, is the existence of a high number of at risk groups, which include, debilitated patients, the immune compromised, ICU patients and surgery patients. Secondly, is the availability of efficient mechanisms of transmission from patient to patient (chains of transmission).

Enterococci have intrinsic resistance to many commonly used antimicrobial agents, including cephalosporins and the semisynthetic penicillinase-resistant penicillins (e.g., oxacillin) and clinically achievable concentrations of clindamycin and aminoglycosides. Compared with streptococci, most enterococci are relatively resistant to penicillin, ampicillin, and the ureidopenicillins. Recent data suggest that this property may not be inherent, but rather acquired after exposure to antibiotics (Hodges et al., 1992). This poses a great challenge to therapy of enterococcal infections.

The buildup and spread of antibiotic resistance determinants among enterococci, to the point where some clinical isolates are resistant to all standard therapies, highlights both the vulnerability of our present armament as well as the looming prospect of a “post-antibiotic era” (where antibiotics lose their current usefulness) (Cohen, 1992). This study intends to establish the antibiotic resistance pattern of enterococcal isolates from clinical and non-clinical specimen of patients at Kiambu District Hospital Kenya.

The emergence and spread of antibiotic-resistant enterococci are not fully understood, since striking differences in different geographical areas have been observed. It is therefore important to provide knowledge on the present status of antibiotic resistance and/or susceptibility of enterococci to antimicrobial agents presently in therapeutic use in Kenya. This information is necessary in order to provide a basis for the therapeutic protocol for enterococcal infection in this country.
CHAPTER II

LITERATURE REVIEW

2.1 HABITAT, MICROBIOLOGY AND EPIDEMIOLOGY

Enterococcal pathogenicity was initially addressed at the end of the 19th century by Mac Callum and Hastings (1899), who isolated an organism, from a case of acute endocarditis, and designated it *Micrococcus zymogenes* based on its fermentative properties. The organism was shown to be resistant to desiccation, heating to 60°C, and several antiseptics including carbolic acid and chloroform. It was also found to be lethal when injected intraperitoneally in white mice, and capable of producing endocarditis in a canine model (Mac Callum and Hastings, 1899). The classification of enterococci as group D streptococci dates back to the scheme established by Rebecca Lancefield in the early 1930's (Lancefield, 1933). In 1984, enterococci were given formal genus status after DNA-DNA and DNA-RNA hybridization studies demonstrated a more distant relationship with the streptococci (Schleifer et al., 1984).

Enterococci are generally considered commensals of the gastrointestinal tract of a variety of organisms including man, and are morphologically indistinguishable from other streptococci. They are found in a number of environments, probably because of dissemination in animal excrement and environmental persistence. In humans, typical concentrations of enterococci in stool are up to $10^8$ CFU per gram (Rice et al., 1995). Although the oral cavity and vaginal tract can become colonized, enterococci are recovered from these sites in fewer than 20% of cases. The predominant species inhabiting the intestine varies. In Europe, the United States of America, and the Far East, *Enterococcus faecalis* predominates in some instances and *E. faecium* in others (Rice et al., 1995). Ecologic or microbial factors promoting intestinal colonization are obscure. Several intrinsic features of Enterococcus may allow members of this genus to survive for extended periods of time, leading to its persistence and nosocomial spread. A century later, enterococci are prominent among nosocomial pathogens, ranking second only to *E. coli* in total nosocomial infections, accounting for more than 12% of all cases (NNIS, 1997).

Of 14 or more enterococcal species (Devriese et al., 1993), only *E. faecalis* and *E. faecium* commonly colonize and infect humans in detectable numbers. Infections caused by the genus
Enterococcus (most notably *Enterococcus faecalis*, which accounts for about 80% of all infections) include urinary tract infections, bacteremia, intra-abdominal infections and endocarditis (Huycke *et al*., 1998). Numerous epidemiologic studies have shown that enterococci can be transmitted from person to person in the hospital. The problem of nosocomial enterococcal infection is compounded by multiple drug resistance (MDR). Although exact modes of nosocomial transmission for MDR enterococci are difficult to prove, molecular microbiologic and epidemiologic evidence strongly suggest spread between patients, probably on the hands of health-care providers or medical devices, and between hospitals by patients with prolonged intestinal colonization (Huycke *et al*., 1998). Prior treatment with antibiotics is common in nearly all patients colonized or infected with MDR enterococci. Clindamycin, cephalosporin, aztreonam, ciprofloxacin, aminoglycoside, and metronidazole use is equally or more often associated with colonization or infection with MDR enterococci than vancomycin use. Other risk factors include prolonged hospitalization; high severity of illness score; intra-abdominal surgery; renal insufficiency; enteral tube feedings; and exposure to specific hospital units, nurses, or contaminated objects and surfaces within patient-care areas (Huycke *et al*., 1998).

The emergence and spread of antibiotic-resistant enterococci is not fully understood, since striking differences in different geographical areas have been observed. In the United States of America, the emergence of *Enterococcus faecium* as a nosocomial pathogen started in the 1980s with an increase in resistance to ampicillin (Bonten *et al*., 2001; Shepard and Gilmore, 2002). Acquisition of vancomycin resistance by a few of these ampicillin-resistant *E. faecium* (AREF) clones, and further dissemination of specific vancomycin resistance transposons to multiple genetic backgrounds, led to an increase in vancomycin-resistant enterococci (VRE) in the following decade (Hanrahan *et al*., 2000). As vancomycin frequently represents the last available therapeutic agent for multiple antibiotic resistant enterococci, the rapid increase in vancomycin resistance indicates that enterococcal infection will pose an increasing therapeutic challenge.

Data compiled for approximately 15,000 isolates over a 3 year period (1995-1997) showed that while resistance to ampicillin and vancomycin is relatively uncommon among *E. faecalis* isolates (<2%), *E. faecium* showed a general trend towards increasing resistance to both ampicillin (83%) and vancomycin (52%) (Huycke *et al*., 1998). Resistance alone, however, does not explain the pervasiveness of enterococci in nosocomial infections. *E. faecalis*, while remaining sensitive to
vancomycin and ampicillin, continues to be the most frequently encountered enterococcal isolate, accounting for 79% of enterococcal infections (Huycke et al., 1998). Reasons for the disparity in the number of infections caused by *E. faecalis* and *E. faecium* are not well known. One explanation for the over representation of *E. faecalis* among clinical isolates may simply relate to natural abundance. Several studies indicate that *E. faecalis* is more abundant in the human gastrointestinal tract (Benno, *et al.*, 1986). An alternative explanation is that the predominance of *E. faecalis* infections is attributable to enhanced virulence, a prospect for which there is some evidence (Huycke *et al.*, 1998).

Enterococci are exceedingly hardy. They tolerate a wide variety of growth conditions, including temperatures of 10°C to 45°C, and hypotonic, hypertonic, acidic or alkaline environments. Sodium azide and concentrated bile salts, which inhibit or kill most microorganisms, are tolerated by enterococci and used as selective agents in agar-based media. *E. faecalis* is able to grow in 6.5% NaCl, at temperatures ranging from 10-45°C, and can survive 30 minutes at 60°C. The earliest descriptions of the organism noted that it was "hardy and tenacious of life" (MacCallum and Hastings, 1899). *E. faecalis* were observed to adapt to the presence of lethal levels of bile salts and detergents, such as sodium dodecyl sulfate (SDS), when first cultured at sub-lethal levels for as little as five seconds (Flahaut *et al.*, 1996). The ability of enterococci to adapt and persist in the presence of detergents may allow them to survive inadequate cleaning regimens, contributing to their persistence in the hospital.

As facultative organisms, enterococci grow under reduced or oxygenated conditions. Enterococci are usually considered strict fermenters because they lack a Kreb’s cycle and respiratory chain (Willett *et al.*, 1992). *E. faecalis* is an exception since exogenous hemin can be used to produce d, b, and o type cytochromes (Huycke *et al.*, 1998). In a survey of 134 enterococci and related streptococci, only *E. faecalis* and *Lactococcus lactis* expressed cytochrome-like respiration. Cytochromes provide a growth advantage to *E. faecalis* during aerobic growth. *E. faecalis* cytochromes are only expressed under aerobic conditions in the presence of exogenous hemin (Huycke *et al.*, 1998) and therefore may promote the colonization of unsuitable sites.
2.2 PATHOGENIC MECHANISMS

In order to infect, enterococci must first be able to colonize, primarily at mucosal surfaces. From the site of colonization, the organism must then evade the host clearance, and ultimately produce pathologic changes in the host, either through direct toxic activity, or indirectly by inducing an inflammatory response.

2.2.1 Colonization and Translocation

Enterococci normally colonize the gastrointestinal tract of man. A close association is likely to exist between enterococci and its host; otherwise the organism would be eliminated due to normal intestinal motility. To colonize the lower bowel, enterococci must survive transit through the low pH of the stomach. Flahaut et al. (1997) demonstrated that exposure of *E. faecalis* to a sub-lethal pH (pH 4.8) for 15-30 minutes protected the organism from a normally lethal challenge at pH 3.2. Suessmuth et al. (1998) have shown that an *E. faecalis* mutant defective in F1 -F0 H+-ATPase activity was unable to grow at pH<6. The H+-ATPase is used to regulate the cytoplasmic pH of *E. faecalis* by proton extrusion. This enzyme has been shown to be activated at low pH. It is therefore apparent that enterococci possess the ability to withstand the low gastric pH, which would facilitate colonization (Hancock and Gilmore, 2002). This attribute may be critical in the ability of multi-drug resistant enterococcal strains to colonize the intestinal tract and cause hospital ward outbreaks. Therapy with antibiotics possessing little anti-enterococcal activity is a key predisposing factor leading to enterococcal colonization and infection. Studies in mice with antibiotic- induced intestinal *E. faecalis* overgrowth demonstrated that organisms can adhere to epithelial surfaces of the ileum, cecum, and colon. These same studies showed that enterococci possess the ability to translocate from the intestinal lumen to the mesenteric lymph nodes, liver and spleen (Tsuchimori et al., 1994). As prior antibiotic therapy appears to be a predisposing factor for enterococcal infection, antibiotic-induced intestinal overgrowth by *E. faecalis*, followed by translocation of the organism into the circulation may offer one explanation for bacteremias of unknown aetiology. The mechanisms responsible for enterococcal translocation are not clearly defined. One hypothesis is that enterococci are phagocytosed by tissue macrophages or intestinal epithelial cells, and are transported across the intestinal wall to the underlying lymphatic system. Failure to kill the phagocytosed organisms could then lead to systemic spread (Hancock and Gilmore, 2002). Olmsted et al. (1994) examined the role of the
plasmid-encoded surface protein, aggregation substance, in the ability of *E. faecalis* to be internalized by cultured intestinal epithelial (HT-29) cells. The presence of aggregation substance significantly augmented *E. faecalis* internalization by HT-29 cells. However, in contrast to the one order of magnitude in enhanced uptake efficiency conferred by aggregation substance, a difference of three orders of magnitude was observed between various enterococcal strains tested. This indicated that additional unknown features of this species play major roles as determinants of internalization efficiency.

The vast majority of *E. faecalis* produce superoxide (O$_2^-$), whereas *E. faecium* isolates do so less frequently. When clinical and commensal isolates of *E. faecalis* were compared for O$_2^-$ production, strains associated with bacteremia produced O$_2^-$ in vitro at a rate 60% higher than stool isolates. What role, if any, superoxide production plays in enterococcal pathogenesis is however not well defined. Hancock and Gilmore (2002), propose that enterococcal strains capable of producing O$_2^-$ are better adapted physiologically to utilize limited resources in the intestinal environment, leading to overgrowth of the organism. Alternatively, O$_2^-$ production may enhance niche control in proximity to the intestinal epithelium. The membrane damaging effects of oxygen radicals may then potentiate the ability of the organism to translocate across a weakened epithelial barrier (Hancock and Gilmore, 2002). Antibiotics lacking substantial anti-enterococcal activity i.e. those that do not deleteriously affect indigenous enterococci, are important predisposing factors for infection. These infections are frequently caused by multiply resistant enterococcal isolates that have been exogenously acquired and appear to have out competed indigenous enterococci in the absence of direct selection. The numbers of exogenously acquired, multiply resistant enterococci would be expected to be minuscule compared to the numbers of indigenous enterococci that are present and well positioned to occupy any niche suitable for enterococcal colonization (Hancock and Gilmore, 2002). The fact that exogenous, multiply resistant, nosocomially transmitted enterococci efficiently colonize the gastrointestinal tract suggests that they may not compete directly for the same niche as indigenous strains. A novel surface protein capable of enabling enterococci to colonize a new area of the gastrointestinal tract, an area perhaps less endowed with immune clearance mechanisms, may explain the ability of these outbreak strains to efficiently colonize and cause disease (Hancock and Gilmore, 2002).
2.2.2 Bacteremia

Nosocomial surveillance data for the period October 1986-April 1997 list enterococci as the third most common cause of nosocomial bacteremia, accounting for 12.8% of all isolates (NNIS, 1997). The translocation of enterococci across an intact intestinal epithelial barrier is thought to lead to many bacteremias with no identifiable source. Other identifiable sources for enterococcal bacteremia include intravenous lines, abscesses, and urinary tract infections (Jett et al., 1994.). The risk factors for mortality associated with enterococcal bacteremia include severity of illness, patient age, and use of broad-spectrum antibiotics, such as third-generation cephalosporins or metronidazole (Stroud et al., 1996). Huycke et al. (1998) showed that patients infected with hemolytic, gentamicin- resistant E. faecalis strains had a fivefold-increased risk for death within three weeks compared to patients infected with non-hemolytic, gentamicin-susceptible strains. Moreover, mode of treatment was not associated with outcome, discounting the contribution of aminoglycoside resistance to this enhanced lethality of infection. Caballero-Granado et al. (1998) analyzed the clinical outcome, including mortality, for bacteremia caused by Enterococcus spp. with and without high-level gentamicin resistance. Mortality associated with high-level gentamicin resistance (29%) was not significantly different from gentamicin-susceptible strains (28%). In addition, these workers found no significant difference in the length of hospitalization after acquisition of enterococcal bacteremia. Taken together, these studies suggest that high-level aminoglycoside resistance does not affect clinical outcome, and that the presence of the E. faecalis cytolysin (hemolysin) may enhance the severity of the infection.

2.2.3 Urinary Tract Infection

Enterococci have been estimated to account for 110,000 urinary tract infections (UTI) annually in the United States (Huycke et al., 1998). Kreft et al. (1992) showed a potential role for the plasmid-encoded aggregation substance in the adhesion of enterococci to renal epithelial cells. E. faecalis harboring the pheromone responsive plasmid pAD1, or various isogenic derivatives, were better able to bind to the cultured pig renal tubular cell line, LLC-PK, than plasmid free cells. Their findings also showed that a synthetic peptide containing the fibronectin motif, Arg-Gly-Asp-Ser, could inhibit binding. Guzman et al. (1989.) analyzed strains of E. faecalis isolated from either urinary tract infections or endocarditis, for their ability to adhere to urinary tract (UT) epithelial cells and the Girardi heart cell line. UTI isolates adhered to the urinary tract epithelial cells in vitro, whereas strains from endocarditis adhered efficiently to the Girardi heart cell line.
A key observation from these experiments was that growth in pooled human serum enhanced the binding of UTI isolates to the Girardi heart cell line (8-fold increase). The authors noted that the serum-dependent alterations to cell adhesion were lost by several sub-cultures in brain heart infusion broth. In a later study, *E. faecalis* adherence was found to be mediated by carbohydrate antigens present on the cell surface (Guzman *et al.*., 1991). Thus, the nature of the interaction of enterococci with uroepithelial tissue appears to be quite complex, involving surface adhesins of protein and/or carbohydrate nature.

### 2.2.4 Endocarditis

Of the diverse infections caused by enterococci, infective endocarditis (IE) is one of the most therapeutically challenging. Enterococci are the third leading cause of infective endocarditis, accounting for 5-20% of cases of native valve IE, and 6-7% of prosthetic valve endocarditis. As noted above, enterococci cultured in serum exhibit enhanced binding to Girardi heart cells. This interaction is inhibited by periodate treatment of the bacterial cell as well as competitive inhibition of binding, by prior incubation of the target cells with specific sugar residues, including D-galactose and L-fructose (Guzman *et al.*., 1991). This suggests that a carbohydrate antigen mediates the adherence of enterococci to cultured heart cells which were derived from the right auricular appendage (Girardi heart). The presence of the pheromone-responsive plasmid pAD1 enhances vegetation formation in enterococcal endocarditis (Chow *et al.* 1993).

### 2.2.5 Endophthalmitis

Colonization of host tissue may play a role in the pathogenesis of endophthalmitis. Enterococci are among the most destructive agents that cause this post-operative complication of cataract surgery. Experiments designed to determine whether aggregation substance targeted *E. faecalis* to alternate anatomical structures within the eye showed that enterococci attach to membranous structures in the vitreous, but that such adherence is not dependent on the presence of aggregation substance (Hancock and Gilmore, 2002).

### 2.2.6 Immune Evasion

For enterococci to maintain an infection, they must successfully evade both specific and nonspecific host defense mechanisms. Other gram-positive pathogens possess attributes, which allow them to survive in the host in spite of powerful nonspecific host defenses mediated
primarily by professional phagocytes, i.e. neutrophils, monocytes, and macrophages. These factors include antiphagocytic polysaccharide capsules, antiphagocytic surface proteins, such as the group A streptococcal M protein, and various secreted toxins with direct toxicity for phagocytic cells.

Harvey et al. (1992) concluded that "neutrophil mediated killing of enterococci was largely a function of complement with antibody playing a less essential but potentially important role." The study also tested several defined virulence traits, which included gelatinase, cytolsin, and aggregation substance of E. faecalis and found no significant correlation between a given trait and resistance to phagocytosis.

The authors identified a strain of E. faecium that exhibited increased resistance to phagocytosis. They were able to detect electron-dense clumps adjacent to the cell wall consistent with the presence of capsular material. It was noted, however, that similar electron-dense clumps surrounded the cell wall of strains of E. faecalis that appeared to be sensitive to Polymorphonuclear-mediated clearance. The nature of the material providing protection to phagocytosis for the E. faecium strain was shown to be protease resistant and periodate sensitive, implicating a carbohydrate (Hancock and Gilmore, 2002). Additional evidence for the importance of anti-enterococcal antibodies in promoting clearance by opsonophagocytic killing was reported by Gaglani et al. (1997). These authors compared killing efficacy by neutrophils using normal human serum, hypogammaglobulinemic serum, or normal human serum adsorbed with the homologous bacterial strain. Their findings suggest that normal human serum has sufficient anti-enterococcal antibodies to promote more than 90% reduction of the bacterial inoculum with serum concentrations as low as 0.5%. Hypogammaglobulinemic serum also promoted > 90% reduction of the bacterial inoculum, but only at serum concentrations above 5%. These findings indicate that the normal human host possesses antibodies to enterococci that aid in opsonophagocytic killing, which might be expected for a normal bowel organism in constant association with the host. It has been observed that enterococci possess the ability to survive within professional phagocytes. Preliminary evidence indicates an important role for aggregation substance in the adherence, entry, and survival in macrophages (Hancock and Gilmore, 2002).
2.2.7 Pathologic Tissue Damage

Following adhesion to host cell surfaces, and evasion of the host immune response, the last step in the pathogenesis of infection is the production of pathologic changes in the host. Such changes can be induced by the host inflammatory cascade or by direct tissue damage as a result of secreted toxins or proteases. Both mechanisms have been observed in studies of \textit{E. faecalis} pathogenesis (Hancock and Gilmore, 2002)

2.2.8 Indirect Tissue Damage

Enterococcal lipoteichoic acid (LTA), also known as the group D streptococcal antigen, has been implicated in a variety of biological processes (Hancock and Gilmore, 2002). Some properties ascribed to LTA include modulation of the host immune response as well as mediating the adherence of enterococci to host cells. Bhakdi \textit{et al.} (1991) found the LTA from enterococci to be as inflammatory as lipopolysaccharide of gram-negative bacteria and may also contribute to the ability of enterococci to exchange and rapidly disseminate genetic determinants.

Ike \textit{et al.} (1984) examined the role of LTA and aggregation substance (AS) in cardiac infections. Strains of \textit{E. faecalis} defective in AS and the enterococcal binding substance (EBS), which is at least partially derived from LTA, did not induce clinical signs of illness when injected to rabbits intraventricularly at levels of $10^8$ cfu/ml. However, EBS+AS- or EBS-AS+ strains induced signs of illness and pericardiac inflammation. All rabbits injected with the EBS+AS+ strain developed illness and died. Surprisingly little inflammation was observed in rabbits injected with the EBS+AS+ strain despite the lethality observed. The authors state that such observations are consistent with the presence of a super antigen. The presence of LTA, EBS and AS together may mediate effects on the host immune response that differ from those seen when either component acts alone.

2.2.9 Direct Tissue Damage

Enterococcal cytolysin and zinc metalloprotease (gelatinase) are secreted factors well suited to contribute to disease severity (Chow \textit{et al.}, 1993). The role of enterococcal cytolysin in disease pathogenesis has been well established (Huycke \textit{et al.}, 1991). It is a unique bacterial toxin that is distantly related to antibiotic bacteriocins, a family of small, post-translationally modified
antimicrobial peptides (Hancock and Gilmore, 2002) and possesses both toxin and bacteriocin activities. It is enriched in clinical isolates, and occurs at a frequency of 45-60% (Coburn et al., 1997), and may provide several levels of selective advantage for *E. faecalis* strains expressing this trait. The presence of the cytolytic phenotype has been suggested to promote the appearance of enterococci in the blood when compared to a non-cytolytic isogenic mutant in a mouse model of septicemia (Stroud et al., 1996). A number of studies support the role for cytolysin in enterococcal infection, both in humans, and animal models (Ike et al., 1987; Chow et al., 1993). The most direct and quantitative evidence for pathologic damage attributable to the cytolysin has been obtained using a rabbit model of endophthalmitis in which natural aberrations in the intraocular immune response, allow a robust infection to be established with as few as 10 organisms (Hancock and Gilmore, 2002). This limited response provides the offending bacterium an opportunity to adapt to *in vivo* growth conditions and environmental cues. A role for the cytolysin in tissue pathology was unambiguously demonstrated both by a reduction in retinal function as measured by electroretinography (B-wave response), and complete destruction of retinal architecture by 24 hours post infection. The contribution of cytolysin to the severity of disease has also been observed in animal models of systemic disease and endocarditis (Huycke et al., 1991). These findings conclusively demonstrate the importance of the *E. faecalis* cytolysin as a major virulence factor in *E. faecalis* infections. However, the cytolytic phenotype is a variable trait typically encoded on pheromone-responsive plasmids. In about half of *E. faecalis* infections, the cytolysin is absent, emphasizing the importance of other traits in pathogenicity (Ike et al., 1987).

The enterococcal gelatinase may also play a measurable role in systemic disease (Dupont et al., 1998) as well as in a caries model using germ-free rats (Hancock and Gilmore, 2002). Dupont et al. (1998) showed a reduced LD50 for mice injected with gelatinase producing (Gel+) strains. Using germ-free rats, Gold et al. (1975) showed that a proteolytic (Gel+) strain exhibited cariogenic activity, whereas three non-proteolytic strains exhibited little cariogenecity. Although demonstration of the involvement of gelatinase in tissue pathology and virulence requires the comparison of isogenic mutants, *in vitro* targets of this enzyme do provide clues for a potential role (Stroud et al., 1996). The gene for gelatinase, *gelE*, encodes an enzyme that shares significant homology with neutral proteinases from Bacillus species and elastase from *Pseudomonas aeruginosa* (Stroud et al., 1996). The proposed targets of this enzyme include
gelatin, casein, hemoglobin, and other bioactive peptides, including the *E. faecalis* sex pheromones. The observation that enterococcal pheromones are potent chemoattractants and that gelatinase can cleave these and other bioactive peptides indicates that gelatinase at least has the potential to modulate the host response to enterococcal infection.

2.3 **ANTIMICROBIAL RESISTANCE**

Enterococci are intrinsically resistant to many antibiotics. Unlike acquired resistance and virulence traits which are usually transposon or plasmid encoded, intrinsic resistance is based in chromosomal genes, which typically are nontransferable (Huycke *et al.*, 1998). Penicillin, ampicillin, piperacillin, imipenem and vancomycin are among the few antibiotics that show consistent inhibitory, but not bactericidal activity against *E. faecalis* (Huycke *et al.*, 1998). *E. faecium* are less susceptible to β-lactam antibiotics than *E. faecalis* because the penicillin-binding proteins (PBPs) of the former have markedly lower affinities for the antibiotics. The first reports of strains highly resistant to penicillin began to appear in the 1980s. Enterococci often acquire antibiotic resistance through exchange of resistance-encoding genes carried on conjugative transposons, pheromone-responsive plasmids, and other broad host-range plasmids (Rice *et al.*, 1995). There many reports of a rapid emergence of MDR enterococci. High-level gentamicin resistance first occurred in 1979 and was quickly followed by numerous reports of nosocomial infection in the 1980s (Huycke *et al.*, 1998). Simultaneously, sporadic outbreaks of nosocomial *E. faecalis* and *E. faecium* infection appeared with penicillin resistance due to β-lactamase production (Murray *et al.*, 1991). Finally, MDR enterococci that had lost susceptibility to vancomycin were reported in Europe and the United States (Sahm *et al.*, 1989). Among several phenotypes for vancomycin resistant enterococci, VanA (resistance to vancomycin and teicoplanin) and VanB (resistance to vancomycin alone) are the most common (Arthur and Courvalin, 1993). In the United States, VanA and VanB account for approximately 60% and 40% of vancomycin-resistant enterococci (VRE) isolates, respectively. Inducible genes encoding these phenotypes alter cell wall synthesis and render strains resistant to glycopeptides. VanA and VanB types of resistance are primarily found among enterococci isolated from clinical, veterinary, and food specimens, but not other common intestinal or environmental bacteria (Klare *et al.*, 1995). In the laboratory, however, these genes can be transferred from enterococci to other bacteria. For example, *Staphylococcus aureus* has been rendered vancomycin-resistant through apparent transfer of resistance from *E. faecalis* on the surface of membrane filters and on
the skin of hairless obese mice (Noble et al., 1992), which indicates that there is no biologic barrier to the emergence of vancomycin-resistant *S. aureus*. Clinical isolates of highly vancomycin-resistant *S. aureus* have yet to be identified, although strains with reduced susceptibility to vancomycin have appeared (Hiramatsu et al., 1997).

Most enterococci have naturally occurring or inherent resistance to various drugs, including cephalosporins and the semisynthetic penicillinase-resistant penicillins (e.g., oxacillin) and clinically achievable concentrations of clindamycin and aminoglycosides. Compared with streptococci, most enterococci are relatively resistant to penicillin, ampicillin, and the ureidopenicillins, with MICs of 1 µg/ml to 8 µg/ml for most *Enterococcus faecalis* and even higher for most *E. faecium*. Many enterococci are also tolerant to the killing effects of cell-wall active agents, including ampicillin and vancomycin. This property may not be inherent, but rather acquired after exposure to antibiotics (Hodges et al., 1992). Inherent *in-vivo* resistance of *E. faecalis* to trimethoprim-sulfamethoxazole may explain the lack of efficacy in animal models. *In-vitro*, trimethoprim-sulfamethoxazole readily inhibits most enterococci at low concentrations, but this activity is lessened by exogenous folates (Zervos et al., 1985). In animal models, this combination has not shown good activity and is not generally accepted as an effective antienterococcal therapy, especially for systemic infections (Chenoweth et al., 1990; Grayson et al., 1990).

In addition to natural resistance to many agents, enterococci have also developed plasmid- and transposon-mediated resistance to tetracycline (as well as minocycline and doxycycline), erythromycin (plus the newer compounds azithromycin and clarithromycin), chloramphenicol, high levels of trimethoprim, and high levels of clindamycin (Murray, 1998).

The propensity of *E. faecalis* to acquire multiple antibiotic-resistance traits may result from a variety of distinctly different mechanisms for conjugation, i.e. bacterial mating. The best studied system of conjugation involves oligopeptides called pheromones and pheromone-responsive plasmids (Murray, 1998). Briefly, strains of *E. faecalis* typically secrete into the culture medium a number of different small peptide sex pheromones specific for different types of plasmids. When a cell containing a pheromone-responsive plasmid (the potential donor cell) comes into contact with its corresponding pheromone, transcription of a gene on the plasmid is turned on, resulting in the synthesis of a sticky substance (called aggregation substance) on its surface.
When the donor cell bumps into another *E. faecalis* aggregation substance, which contains two Arg-Gly-Asp motifs, it sticks to the binding substance on the surface of most *E. faecalis* cells, causing them to clump together. In the test tube, clumps of cells actually fall to the bottom of the tube, resulting in a visible aggregate. By a process not yet well understood, the pheromone-responsive plasmid can then transfer from the donor bacterium to the other (recipient) bacterium. Once the recipient cell has acquired this particular plasmid, the synthesis of the corresponding sex pheromone is shut off to prevent self-clumping. This system of conjugation, which occurs primarily in *E. faecalis*, is highly efficient and results in transfer of plasmids in both filter and broth matings (Murray, 1998).

### 2.4 MECHANISMS OF RESISTANCE

#### 2.4.1 β-Lactam Resistance

Complete or relative resistance to β-lactams is a characteristic feature of the genus Enterococcus. *E. faecalis* is typically 10 to 100 times less susceptible to penicillin than are most streptococci, while *E. faecium* is at least 4 to 16 times less susceptible than *E. faecalis*. While most isolates of *E. faecalis* are inhibited by concentrations of penicillin or ampicillin (1 to 8 µg/ml) easily achievable in humans, isolates of *E. faecium* usually require an average of 16 to 64 µg/ml to inhibit growth, although some isolates are even more resistant (Murray, 1997). An additional problem with enterococci is that they are typically tolerant to β-lactams (i.e., MBC/MIC of >32). The major mechanism underlying this resistance has been the production of low-affinity PBPs. β-Lactamase-producing enterococci are infrequently isolated. Unlike most staphylococci, where β-lactamase production is inducible, β-lactamase production in enterococci is constitutive, low level, and inoculum dependent (Yesim *et al*, 2000).

#### 2.4.2 Aminoglycoside Resistance

Moellering and Weinberg, (1971) demonstrated that two types of streptomycin resistance occur in enterococci: (i) moderate-level resistance (MIC, 62 to 500 µg/ml), because of low permeability, which can be overcome with a penicillin (which increases the cellular uptake of the aminoglycoside); and (ii) high-level resistance (MIC, ≥2,000 µg/ml), which is either ribosomally
mediated or due to the production of aminoglycoside-inactivating enzymes. Since enterococcal resistance to gentamicin and streptomycin occur by different mechanisms, it is important to test susceptibilities to both agents. Gentamicin resistance is predominantly the result of the presence of the inactivating enzyme 2"-phosphotransferase-6'-acetyltransferase conferring resistance to gentamicin, tobramycin, netilmicin, amikacin, and kanamycin. Hence, gentamicin resistance is a good predictor of resistance to other aminoglycosides except streptomycin. Streptomycin resistance is encountered mainly in enterococcal strains that produce streptomycin adenyltransferase; these strains remain susceptible to gentamicin. Penicillin-aminoglycoside synergy does not occur in high-level aminoglycoside-resistant enterococci (streptomycin MIC, ≥2,000 µg/ml: gentamicin MIC, ≥ 500 µg/ml) (Yesim et al., 2000).

### 2.4.3 Vancomycin Resistance

There are five recognized phenotypes of vancomycin resistance, VanA, VanB, VanC, VanD, and VanE (Arthur and Courvalin, 1993). VanA and VanB resistance phenotypes were described primarily in *E. faecalis* and *E. faecium*. VanA-resistant strains possess inducible, high-level resistance to vancomycin (MICs, ≥64 µg/ml) and teicoplanin (MICs, ≥16 µg/ml) (Arthur and Courvalin, 1993). Resistance can be induced by glycopeptides (vancomycin, teicoplanin, avoparcin, and ristocetin) and by non-glycopeptide agents such as bacitracin, polymyxin B, and robenidine, a drug used to treat coccidial infections in poultry. The VanC resistance phenotype was described in *E. casseliflavus* and *E. gallinarum*, which demonstrate intrinsic, low-level resistance to vancomycin (MICs, 4 to 32 µg/ml) and are susceptible to teicoplanin (Yesim et al., 2000).

Under normal conditions of peptidoglycan synthesis in enterococci, two molecules of D-alanine are joined by a ligase enzyme to form D-Ala-D-Ala, which is then added to UDP-N-acetylmuramyl-tripeptide to form the UDP-N-acetylmuramyl-pentapeptide. The UDP-N-acetylmuramyl-pentapeptide, when incorporated into the nascent peptidoglycan (transglycosylation), permits the formation of cross-bridges (transpeptidation) and contributes to the strength of the peptidoglycan layer (Eliopoulos, 1997). Vancomycin binds with high affinity to the D-Ala-D-Ala termini of the pentapeptide precursor units, blocking their addition to the growing peptidoglycan chain and preventing subsequent cross-linking (Yesim et al., 2000).
This phenotypic classification scheme is useful, because it usually corresponds well to the genotypic classification and utilizes information that can be derived simply and inexpensively in a laboratory. Each of these classes of resistance is further explained below.

2.4.3.1 VanA glycopeptide resistance.

The vanA gene and other genes involved in the regulation and expression of vancomycin resistance (vanR, vanS, vanH, vanX, and vanZ) are located on a transposon (Tn1546) of *E. faecium*, which often resides on a plasmid (Arthur *et al.*, 1993). Expression of these genes results in the synthesis of abnormal peptidoglycan precursors terminating in D-Ala-D-lactate instead of D-Ala-D-Ala. Vancomycin binds to D-Ala-D-Lac with markedly lower affinity than it does to the normal dipeptide product (Bugg *et al.*, 1991). VanA alone cannot confer resistance to vancomycin, probably because D-hydroxy acids such as D-Lac are neither natural products present in the environment of enterococci nor normally produced by enterococci. Thus, to synthesize D-lactate, enterococci must acquire the gene(s) within the vanA operon required to produce the substrate for VanA. VanH is responsible for the synthesis of D-lactate.

VanR and VanS proteins constitute a two-component regulatory system that regulates the transcription of the vanHAX gene cluster. VanS apparently functions as a sensor to detect the presence of vancomycin or, more likely, some early effect of vancomycin on cell wall synthesis. VanS then signals VanR, the response regulator, which results in activation, or turning on, of the synthesis of some other proteins (VanH, VanA, and VanX) involved in resistance (Baptista *et al.*, 1996).

Cross-linking of the precursors to the growing peptidoglycan is processed in bacteria by the PBPs. The replacement of D-Ala by D-Lac does not impair cross-linking of the modified precursors to the growing peptidoglycan chain. These high-molecular-weight PBPs display a higher affinity for β-lactams. Since VanA resistance is inducible, a shift in the PBPs occurs only in the presence of vancomycin and results in β-lactam hypersusceptibility. This effect explains the synergy displayed by the combination of the two classes of drugs against vancomycin-resistant strains (Yesim *et al.*, 2000).
2.4.3.2 VanB glycopeptide resistance.

VanB glycopeptide resistance in enterococci is mediated by an abnormal ligase (VanB) that is structurally related to VanA ligase (76% amino acid identity). VanB protein also favors the production of the pentadepsipeptide terminating in D-Ala-D-Lac (Evers et al., 1993). Genes analogous to their class A resistance counterparts are designated vanHB, vanXB, vanYB, vanRB, and vanSB. Levels of D,D-dipeptidase activity (VanXB) correlate with levels of vancomycin resistance. The regulatory system in class B strains appears insensitive to induction by teicoplanin. Teicoplanin induces the synthesis of VanA-related proteins but does not induce the production of VanB-related proteins. On the other hand, vancomycin induces the synthesis of the resistance proteins of both systems, and in fact, if a teicoplanin-susceptible enterococcus with the vanB gene cluster is pre-exposed to vancomycin, the strain then tests teicoplanin resistant as well. In addition, teicoplanin-resistant mutants can be derived from teicoplanin-susceptible, vanB-containing enterococci when these organisms are plated onto teicoplanin-containing agar. Such mutants can also arise in vivo during therapy (Murray, 1997). Possible mechanisms for teicoplanin resistance of these mutants include the loss of their requirement for an inducer (that is, if they constitutively produce high levels of the vancomycin resistance proteins) and the ability of teicoplanin to act as an inducer.

Another difference between VanA- and VanB-type resistances is that VanA is more widely distributed. The vanA ligase gene has also been found in a wider range of enterococcal species as well as in Corynebacterium spp., Arcanobacterium haemolyticum, and Lactococcus spp., while vanB has been found primarily in E. faecium and E. faecalis. The difference in the dissemination of these resistance traits may be related to the observation that the vanA gene cluster is often located on a transposon similar to Tn1546, which, in turn, can be a part of a conjugative (transferable) plasmid (Arthur et al., 1993, Yesim et al., 2000). Such a genetic arrangement is an excellent avenue for the dissemination of these genes. The vanB cluster is often located on the host chromosome and initially was thought not to be transferable to other bacteria. However, it can also occur on plasmids, and, even when it is chromosomal, this gene cluster has been transferable as part of large mobile elements, perhaps related to large conjugative transposons (Quintiliani and Courvalin, 1994).
2.4.3.3 VanC glycopeptide resistance.

Low-level resistance to vancomycin is typical of *E. gallinarum*, *E. casseliflavus*, and *E. flavescens*. The nucleotide sequences of the vanC-1 gene in *E. gallinarum*, the vanC-2 gene in *E. casseliflavus*, and the vanC-3 gene in *E. flavescens* have been reported (Clark *et al.*, 1998). VanC ligase of *E. gallinarum* favors the production of a pentapeptide terminating in D-Ala-D-Ser. Substitution of D-Ser for D-Ala is presumed to weaken the binding of vancomycin to the novel pentapeptide. D,D-Dipeptidase and D,D-carboxypeptidase activities analogous to those of VanA and VanB strains occur. The level of resistance expressed represents the balance achieved between normal and abnormal peptidoglycan synthesis (Eliopoulos, 1997). The presence of variable amounts of D-Ala-D-Ala relative to D-Ala-D-Ser could account for the variable levels of vancomycin resistance observed among isolates of VRE carrying the VanC phenotype. That is, lower MICs could be explained by the presence of larger amounts of D-Ala-D-Ala, which enables vancomycin to inhibit cell wall synthesis, and higher MICs could be explained by a higher proportion of D-Ala-D-Ser (Eliopoulos, 1997). Resistance may be inducible or constitutive (Dukta-Malen *et al.*, 1994).

2.4.3.4 VanD glycopeptide resistance.

A novel vancomycin resistance gene designated vanD was first described in a New York Hospital in 1991. The strain carrying this resistance trait was an *E. faecium* strain that was inhibited by vancomycin at 64 µg/ml and teicoplanin at 4 µg/ml. Partial sequencing of the ligase gene showed that it was distinct from but similar to the vanA and vanB ligase genes (Perichon *et al.*, 1997). Three clinical isolates of vancomycin-resistant *E. faecium* carrying the VanD resistance trait were found in Boston, and the deduced amino acid sequence of VanD showed 67% identity to those of VanA and VanB. VanD appears to be located on the chromosome and is not transferable to other enterococci (Yesim *et al.*, 2000).

2.4.3.5 VanE glycopeptide resistance.

The VanE vancomycin resistance gene has been described in *E. faecalis*, which is resistant to low levels of vancomycin (MIC, 16 µg/ml) and susceptible to teicoplanin (MIC, 0.5 µg/ml). This
new resistance phenotype has similarities to the intrinsic VanC type of resistance (Fines et al., 1999).

### 2.4.3.6 Vancomycin-dependent enterococci.

An interesting phenomenon that has developed in some strains of VanA- and VanB-type VRE is that of vancomycin dependence (Dever et al., 1995). These enterococci are not just resistant to vancomycin but require it for growth. Vancomycin-dependent enterococci have been recovered from apparently culture-negative clinical samples by plating them onto vancomycin-containing agar, such as that used for isolation of Campylobacter or gonococci (Yesim et al., 2000). A likely explanation for the phenomenon of vancomycin dependence is that these enterococci turn off their normal production of D-Ala-D-Ala and then can grow only if a substitute dipeptide-like structure is made. With most VanA- and VanB-type enterococci, this occurs only in the presence of vancomycin, which induces the synthesis of associated dehydrogenase (VanH) and ligase (VanA or VanB) that make D-Ala-D-Lac. The reason for the cell turning off the synthesis of D-Ala-D-Ala is that as long as vancomycin is present, D-Ala-D-Ala is not necessary for cell wall synthesis by VRE (Murray BE, 1997). Once the vancomycin is removed, D-Ala-D-Lac is no longer synthesized, and without either D-Ala-D-Ala or D-Ala-D-Lac, the cell cannot continue to grow or replicate. Reversion to vancomycin independence has been observed. It probably occurs by either a mutation that leads to constitutive production of D-Ala-D-Lac or one that restores the synthesis of D-Ala-D-Ala (Yesim et al., 2000).

### 2.5 THERAPEUTIC APPROACHES

Prior to any treatment of hospital acquired infections, all suspected intravenous lines, intra-arterial catheters, and urinary catheters should be removed and abscesses drained. Infections that do not require bactericidal therapy are usually treated with a single antibiotic directed toward enterococci; these infections include urinary tract, intra-abdominal and uncomplicated wound infections. Monotherapy is adequate treatment for many patients with enterococcal bacteremia when no evidence of endocarditis exists. In clinical practice, combination therapy with a cell wall–active agent and an aminoglycoside should be considered for treating serious enterococcal
infections in critically ill patients and in those with evidence of sepsis, as well as in patients with endocarditis, meningitis, osteomyelitis, or joint infections (Caron et al., 1995).

For monotherapy of susceptible *E. faecalis*, ampicillin is the drug of choice. Ampicillin MICs are 2- to 4-fold lower compared with penicillin MICs for most isolates. For rare strains that are resistant to ampicillin because of beta-lactamase production, ampicillin plus sulbactam may be used. For patients with a penicillin allergy or strains with high-level penicillin resistance due to altered PBPs, vancomycin should be administered. Nitrofurantoin is effective for the treatment of enterococcal urinary tract infections, including many caused by VRE strains (Caron et al., 1995).

Combination therapy with a cell wall–active agent (e.g., ampicillin, vancomycin) and an aminoglycoside (e.g. gentamicin or streptomycin) is necessary to adequately treat patients with enterococcal endocarditis. This combination results in synergistic bactericidal activity against susceptible enterococcal strains. At least 4 weeks of combination therapy is recommended for the treatment of enterococcal endocarditis. Six weeks of combination therapy is recommended for patients with symptoms for more than 3 months before starting therapy, for patients who relapsed after shorter courses of therapy, and for patients with prosthetic valves (Fraser, 2006).

If vancomycin is used in the course of treatment for endocarditis, a 6-week rather than 4-week course of therapy is recommended. Combination therapy is also recommended for the treatment of enterococcal meningitis, and treatment is usually administered for at least 2-3 weeks. Intravenous linezolid or intravenous plus intraventricularly quinupristin-dalfopristin are also recommended for meningitis (Fraser, 2006).

The emergence of strains with multidrug-resistant determinants has significantly complicated the management of enterococcal infections. Patients infected with strains that exhibit high-level resistance to ampicillin are treated with vancomycin. Strains with high-level gentamicin resistance should be tested for high-level streptomycin resistance. For gentamicin-resistant strains, the alternative is streptomycin. Treatment options are limited for patients with endocarditis who are infected with strains that exhibit high-level resistance to all aminoglycosides. For *E. faecalis*, prolonged therapy with high doses of ampicillin plus imipenem/cilastatin or ampicillin plus ceftriaxone might be considered. For *E. faecium*, either
Linezolid or quinupristin-dalfopristin may be effective, and daptomycin or tigecycline could be considered (Caron et al., 1995).

For infections caused by VRE, the treatment is based on infection severity and *in vitro* susceptibility of the strain to other antibiotics. Uncomplicated urinary tract infections have been treated successfully with nitrofurantoin. Isolates that remain relatively susceptible to penicillin or ampicillin (MICs of 0.5-2 mcg/mL) may be treated with high doses of these agents. Doxycycline, chloramphenicol, and rifampin in various combinations have been used to treat VRE infections, (Caron et al., 1995).

Quinupristin/dalfopristin is a combination of streptogramins A and B. It targets the bacterial 50S ribosome and results in inhibited protein synthesis. It is available intravenously for the treatment of *E. faecium* infections but is not effective against *E. faecalis* strains (Aumercier et al., 1992). In addition, quinupristin/dalfopristin is bacteriostatic only, potentially allowing emergence of resistance (Chow et al., 1997). For these reasons the drug may have only a limited role in treating MDR enterococcal infections.

Linezolid, an oxazolidinone antibiotic, is available orally and intravenously and is used to treat infections caused by *E. faecium* and *E. faecalis* strains, including VRE (Jones et al., 1996).

Daptomycin, a lipopeptide antibiotic that works by altering the bacterial membrane function, is indicated for the treatment of vancomycin-susceptible *E. faecalis*–complicated skin infections. It became available in 2003, and, although it has in vitro activity against all strains of enterococci, the data regarding its use in *E. faecium* or VRE infections are limited (Fraser, 2006).

Tigecycline is a glycolcycline antibiotic that was released in 2005 and can be used to treat gram-positive, gram-negative, and anaerobic bacterial infections. It can be used to treat vancomycin-sensitive *E. faecalis* infections, and, although, it has in vitro activity against *E. faecium* and VRE (including *E. casseliflavus* and *E. gallinarum*), as with daptomycin, clinical data are limited (Eliopoulos et al., 1994).

Teicoplanin is a glycopeptide that has been shown to have in vitro activity against *E. gallinarum* and *E. casseliflavus*, but not against the most common VanA, type VRE (Fraser, 2006).
Dalbavancin is a new antibiotic that is structurally related to vancomycin and teicoplanin. It is marketed especially for MRSA infections but also has activity against non-VRE enterococci. It has a very long half-life, so weekly administration is possible (Fraser, 2006).

The substantial drawback of the broad-spectrum approach is that the more organisms affected (both protective commensals as well as pathogens), the more opportunities for resistance to evolve. Broad-spectrum antibiotics permit empiric therapy in the absence of a specific diagnosis and generate a more substantial return on investment in the short term. However, broad-spectrum antibiotics affect not only disease causing organisms but also commensals present in numbers large enough to generate resistance by otherwise rare mutations or genetic exchange events. As long as market forces favor development of broad-spectrum therapeutics, a cycle of drug introduction followed by emergence of resistance undoubtedly will continue.

2.6 INFECTION CONTROL

Controlling the spread of MDR enterococci among in-patients is difficult. Little is known about the biology of enterococcal transmission or the specific microbial factors favouring colonization by exogenous enterococcal strains. Nevertheless, VRE infection control policies, which could apply to MDR enterococci, were published by the CDC in 1995. Control methods include routine screening for vancomycin resistance among clinical isolates, active surveillance for VRE in intensive care units, contact isolation to minimize person-to person transmission, and vancomycin restriction. Not all hospitals can or are willing to perform active surveillance. Because more patients are typically colonized with VRE (3% to 47%) than are infected (Handwerger et al., 1993) and because intestinal colonization can be prolonged, passive surveillance by routine cultures allows colonized inpatients to go unidentified and serve as point sources for continued spread of VRE. Even if all colonized inpatients are successfully identified, VRE may be spread by health-care workers through either inadequate hand washing or through contact with items such as bedrails, sinks, faucets, and doorknobs (enterococci can persist for weeks on environmental surfaces) (Noskin et al., 1995). Decontamination efforts must be rigorous. Antibiotics may promote colonization and infection with MDR enterococci by at least two mechanisms. First, many broad-spectrum antibiotics have little or no anti-enterococcal activity, and administration commonly leads to overgrowth of resistant enterococci at sites at risk for infection. Second, most antibiotics substantially reduce the normal resistance of the intestinal
tract to colonization by exogenous organisms (Vollaard and Clasener, 1994). Colonization resistance results primarily from the “limiting action” of the normal anaerobic flora, and to a lesser extent from an intact mucosa, gastric acid secretion, intestinal motility, and intestinal associated immunity (Vollaard and Clasener, 1994). Antibiotic-induced alterations in the protective flora of the intestine provide large footholds for colonization with exogenous pathogens such as MDR enterococci (Vollaard and Clasener, 1994). Antibiotic restriction programs would be more effective if they included prudent prescribing of all antibiotics. At a minimum, a successful program for control of MDR enterococci requires effective passive and active surveillance to identify colonized and infected patients, absolute adherence to contact isolation by health-care workers, rigorous decontamination of patient-contact areas and judicious use or restriction of vancomycin and other broad-spectrum antibiotics.
CHAPTER III - MATERIALS AND METHODS

3.1 Study design
This was a cross-sectional descriptive study.

3.2 Study population
The study population comprised of inpatients attending Kiambu District Hospital and healthy controls who were students of the Kenya Polytechnic University College. Stool and clinical material from inpatients sent to the microbiology laboratory for culture, were used in the study. The clinical material included, blood, CSF, pus swabs, and urine. Stools were obtained from healthy controls.

3.3 Patient data
The gender of the patient and the hospital number, the date and source of the culture was recorded.

3.4 Study area
The study was carried out in the clinical microbiology laboratory of Kiambu District Hospital, Kenya between April and December 2011.

3.5 Sampling method
Systematic random sampling was employed for stool and urine samples. In this method every third sample collected was included in the study. Consecutive samples were taken for all clinical samples other than stool and urine.

3.6 Inclusion exclusion criteria
Inclusion:
1. Samples from consenting healthy controls consisting of students from the Kenya Polytechnic University College who complete a recruitment procedure consent form.
2. Samples from inpatients at Kiambu District Hospital sent to the laboratory for bacterial culture (i.e. stools or other clinical material). These were samples provided by patients for bacterial culture.
Exclusion:
  1. Samples from outpatients and any clinical sample not meant for bacterial culture.
  2. Use of antibiotics within the last month by the patient, for healthy controls
  3. Hospitalization within the last one month for healthy controls.

3.7 Sample size calculation
The aims of this study were to determine antibiotic susceptibility patterns of enterococci and their distribution in various specimen types. A sample size appropriate for determination of distribution was thus used. Sample size was calculated using Fischer’s formula \( n = Z^2 pq/d^2 \) (Daniel, 1995), and applying the nosocomial infection rate of 10%, where ‘n’ is the desired sample size, ‘Z’ is the standard normal deviate at 95% confidence interval (i.e. 1.96), ‘p’ is the prevalence rate of nosocomial infections, ‘q’ is the non-attack rate (1-p), and ‘d’ is the level of statistical error at a significance level of 5% (0.05), bringing the number of samples required to 139. Out of the 139 samples examined, 50 were stool samples from healthy controls and 50 stool samples from patients and 39 clinical samples other than stool.

3.8 Study procedure
Stool, blood, cerebrospinal fluid, pus and urine samples collected from the study population and controls were sent to the microbiology laboratory for culture and sensitivity testing.

3.9 Recruitment procedure for healthy controls
Consenting students of the Kenya Polytechnic university college were recruited by systematic random sampling where every third sample was taken. They were screened using a questionnaire as specified in the appendix IV.

3.10 Isolates
The strains were isolated from stool samples and clinical material in the clinical microbiology laboratory of Kiambu District Hospital. The clinical samples included but were not limited to, blood, cerebrospinal fluid, respiratory tract samples, abdomen samples, pus swabs, and urine. By use of the streak-plate technique each sample was inoculated onto Bile azide agar to separate enterococci from gram-negative bacteria in the sample. Using a sterile loop, a loop-full of the
specimen was spread over a small area at the edge of the plate and then used to streak the whole plate. Single-colony isolates were purified by inoculation into a selective medium (Bile esculin azide) and the subsequent growths (colonies) were inoculated into 5ml of BHI/peptone broth, which were then incubated overnight at 35°C.

3.11 Identification

Strains were identified by gram reaction and biochemical tests. The identification was confirmed by the API 20 STREP system (BioMerieux, Marcy l’Etoile, France). The API 20 STREP system is routinely used to identify the following enterococcus species: Enterococcus casseliflavus, E. durans, E. faecalis, E. faecium, E. gallinarum, E. avium.

3.12 Tests

1. Growth in azide medium (Pfizer selective enterococcus/Bile esculin azide). This medium is selective and is used to separate enterococci from gram-negative bacteria in the sample. The medium contains Sodium azide and/or concentrated bile salts, which inhibit or kill most microorganisms but are tolerated by enterococci and used as selective agents in agar based media.

2. Gram stains. The Gram stain is a differential stain which allows most bacteria to be divided into two groups, Gram-positive bacteria and Gram-negative bacteria. The technique is based on the fact that the Gram positive cell wall has a stronger attraction for crystal violet when Gram's iodine is applied than does the Gram negative cell wall. Gram's iodine is known as a mordant. It is able to form a complex with the crystal violet that is attached more tightly to the Gram-positive cell wall than to the Gram-negative cell wall. This complex can easily be washed away from the Gram-negative cell wall with ethyl alcohol. Gram-positive bacteria, however, are able to retain the crystal violet and therefore will remain purple after decolorizing with alcohol. Since Gram-negative bacteria will be colorless after decolorizing with alcohol, counterstaining with safranin will make them appear pink. Enterococci are gram positive and therefore remain purple after the procedure.

3. Catalase test. This test is useful in differentiating enterococci and staphylococci which are morphologically similar. Enterococci are catalase negative while staphylococci are catalase positive. Many bacteria possess enzymes that afford protection against toxic O₂
products. Obligate aerobes and facultative anaerobes usually contain the enzymes superoxide dismutase which catalyzes the destruction of superoxide, and either catalase or peroxidase, which catalyze the destruction of hydrogen peroxide as follows:

\[
2O_2 + 2H^+ \rightarrow O_2 + H_2O_2
\]

Superoxide dismutase

\[
2H_2O_2 \rightarrow 2H_2O + O_2
\]

Catalase/peroxidase

Catalase production and activity can be detected by adding the substrate H\textsubscript{2}O\textsubscript{2} to a culture. If catalase was produced by the bacteria the chemical reaction will liberate free oxygen.

4. Tolerance to 40% bile esculin. The basis of the esculin test is the hydrolysis of esculin (a glucoside) into glucose and esculetin by a microorganism that has a constitutive (noninducible) \(\beta\)-glucosidase or esculinase enzyme. When esculetin is produced, it reacts with an iron salt in the medium to form a phenolic iron complex which produces a dark brown or black color. If bile is added to the medium, the microorganisms must be able to grow in its presence in order to hydrolyze esculin. The 40% bile in bile esculin medium inhibits most strains of streptococci, but does not inhibit enterococci.

5. Growth in 6.5% NaCl broth (The salt tolerance test). This test can be used to differentiate enterococci from group D streptococci, as enterococci are the only streptococci that will grow in 6.5% NaCl broth.

6. API. The API 20 STREP is a standardized method consisting of 20 biochemical tests that offer wide spread capabilities. It enables group or species identification of most streptococci encountered in medical bacteriology. It consists of a strip of 20 microtubules containing dehydrated substrates for the demonstration of enzymatic activity or the fermentation of sugars. The enzymatic tests are inoculated with a dense suspension of organisms, made from a pure culture, which is used to rehydrate the enzymatic substrates. The metabolic end products produced during the incubation period are either revealed through spontaneous colored reactions or by the addition of reagents. The tests included in the system are as shown in appendix II.
3.13 Inoculation and incubation
Single-colony isolates were inoculated into 5 ml of Brain heart infusion broth/Todd-Hewitt broth, which was then incubated overnight at 35°C. All subsequent test media (for biochemical tests) were inoculated using a Pasteur pipette with 1 drop of the BHI broth culture. All of the tests were incubated at 35°C and were read at 24hrs and if the results were indeterminate they were read again at 48hrs.

3.14 Ethical consideration for healthy controls
This study was approved by the Department of Medical Microbiology of the University of Nairobi. Ethical clearance to conduct the study was obtained from Kenyatta National Hospital/University of Nairobi Ethics and Research committee prior to carrying out any procedure.

3.15 Antimicrobial susceptibility testing
All isolates identified as enterococci were tested by standard disc diffusion, the Kirby-Bauer method on blood agar. Inocula were prepared from overnight growth on BHI/Todd-Hewitt/peptone broth cultures. All plates dispensed with antibiotic discs, were incubated for 18 to 20 hrs at 35°C in ambient air.

All isolates were tested for susceptibility to streptomycin HL (300µg), gentamycin HL (120µg), vancomycin (5µg), ciprofloxacin (5µg), chloramphenicol (30µg), amoxicillin (20µg), ampicillin (2µg) and nitrofurantoin (100µg). In addition, tests for susceptibility to amoxicillin-clavulanate (2:1) amoxicillin-clavulanate (20-10µg) were carried out for strains showing ampicillin resistance.

3.16 Inferential statistical analysis
The Chi Square test was used to test the significance of difference in the resistance patterns between clinical and stool isolates and also between patient and control stool isolates. The test was conducted at α = 0.05 level of significance. Results were considered significant when p < 0.05. The analysis was done on a drug by drug basis. Statistical significance for ampicillin resistance could not be tested using Chi square test because stool isolates returned a zero resistance to this drug. The Chi square test calculation requires that no cell entry in the table has
a count less than one (the test calculation requires a positive integer for validity). Ampicillin resistance was zero for stool isolates. Similarly test of statistical significance of vancomycin resistance could not be performed since no strain showed resistance to this drug.
CHAPTER IV - RESULTS

4.1 Enterococcal species prevalence

A total of 139 participants took part in the study. These consisted of 78 males and 61 females. The 139 participants each provided a samples that was included in the study and was positive for enterococci and from which a single enterococcus species was isolated.

There was no statistical difference in the gender ratio between clinical participants and healthy controls ($\chi^2=2.61$, $p>0.1$). There was also no statistical difference in the gender ratio between clinical participants who provided specimen other than stool and patients who provided stools ($\chi^2=2.04$, $p>0.1$).

Table 1. Distribution of sexes in the study and control groups

<table>
<thead>
<tr>
<th>Category of Participants</th>
<th>Female</th>
<th>Male</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical</td>
<td>24</td>
<td>15</td>
<td>39</td>
</tr>
<tr>
<td>Stools (Patients)</td>
<td>22</td>
<td>28</td>
<td>50</td>
</tr>
<tr>
<td>Stools (Controls)</td>
<td>21</td>
<td>29</td>
<td>50</td>
</tr>
<tr>
<td>Totals</td>
<td>67</td>
<td>72</td>
<td>139</td>
</tr>
</tbody>
</table>

Out of the 139 samples from which enterococcus species were recovered, *E. faecalis* (66, 47.5%) and *E. faecium* (39, 28%) constituted the predominant isolates. Other enterococcal species which were neither *E. faecalis* nor *E. faecium* were 34 (24.5%). See table 2.
Table 2. Overall distribution of enterococcal species in both clinical and non-clinical samples

<table>
<thead>
<tr>
<th></th>
<th>Clinical samples (n=39)</th>
<th>Patient stool (n=50)</th>
<th>Control/healthy Stool (n=50)</th>
<th>Overall prevalence (n=139)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td>32 82%</td>
<td>19 38%</td>
<td>15 30%</td>
<td>66 47.5%</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>6 15.4%</td>
<td>16 32%</td>
<td>17 34%</td>
<td>39 28%</td>
</tr>
<tr>
<td>Other species</td>
<td>1 2.6%</td>
<td>15 30%</td>
<td>18 36%</td>
<td>34 24.5%</td>
</tr>
<tr>
<td>Totals</td>
<td>39</td>
<td>50</td>
<td>50</td>
<td>139 100%</td>
</tr>
</tbody>
</table>

There were 39 non-stool clinical specimens from which Enterococcal species were isolated. These included pus swabs (21), urine (13), blood (2) and ascitic fluid (3). Majority of the specimens (82%) had *E. faecalis*, while 15.4% of the specimens had *E. faecium* and 2.6% had other enterococcal species. See table 3.

Table 3. Distribution of enterococcal species in clinical samples

<table>
<thead>
<tr>
<th></th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td>32</td>
<td>82.1</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>6</td>
<td>15.4</td>
</tr>
<tr>
<td>Other Enterococci species</td>
<td>1</td>
<td>2.6</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>100.0</td>
</tr>
</tbody>
</table>

*E. faecalis* predominated in both pus (90.5%) and urine (61.5%) samples among these clinical isolates. *E. faecalis* was the only species recovered from blood and ascitic fluid. See table 4. Each specimen provided only one isolate since only one pure colony was used in the purification step. There was therefore no overlap where a specimen could provide more than one enterococcal species. None of the five CSF samples cultured during the study period exhibited enterococci.
Table 4. Distribution of enterococcal species by specimen

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ascitic fluid</th>
<th>Blood</th>
<th>Pus swabs</th>
<th>Urine</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecalis</td>
<td>3</td>
<td>2</td>
<td>19</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>E. faecium</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Other species</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Totals</td>
<td>3</td>
<td>2</td>
<td>21</td>
<td>13</td>
<td>39</td>
</tr>
</tbody>
</table>

Unlike the distribution in clinical isolates, *E. faecium* and other enterococcal species were more represented in stool (Table 2). There was no significant difference in the distribution of enterococcus species in stool specimen of patients and healthy controls (p>0.1). *E. faecalis* was isolated from 38% of patients’ stools and from 30% of healthy controls stools. *E. faecium* was isolated from 32% of patients’ stools and from 34% of healthy control stools. Other enterococcal species constituted 30% in patient stool isolates and 36% in healthy control stool isolates. See table 2.

4.2 Antimicrobial susceptibility patterns

4.2.1 Clinical isolates

All clinical enterococcal isolates were susceptible to vancomycin. Resistance of *E. faecium* was higher than that of *E. faecalis* to the following drugs: ampicillin (16.7% vs 6.3%), streptomycin HL (50% vs 34.4%), nitrofurantoin (33.3% vs 15.6%) and amoxicillin (50% vs 15.6%). *E. faecalis* was however more resistant than *E. faecium* to ciprofloxacin (65.6% vs 50%) and to chloramphenicol (53.1% vs 33.3%). See table 5. In this part of analysis, there was only one non-*faecium*, non-*faecalis* species, which was classified as other enterococcal species. That isolate was susceptible to all tested antibiotics.

There was no statistically significant difference in the rate of resistance between *E. faecalis* and that of *E. faecium* from clinical isolates to all the drugs tested, namely ampicillin ($\chi^2=0.002$, p=0.1), gentamycin HL ($\chi^2=0.42$, p=0.1), streptomycin HL ($\chi^2=0.71$, p=0.1), nitrofurantoin ($\chi^2=0.21$, p=0.1), ciprofloxacin ($\chi^2=1.41$, p=0.1), and chloramphenicol ($\chi^2=1.78$, p=0.1). The test was not performed for vancomycin since none of the isolates was resistant to the drug. All
the three isolates resistant to ampicillin were susceptible to amoxicillin-clavulanate. The resistance to gentamycin was relatively similar in *E. faecium* (16.8%) and *E. faecalis* (18.8%).

Overall, resistance was highest to ciprofloxacin (61.5%). The overall resistances to the other antimicrobials were as follows: chloramphenicol (48.7%), streptomycin HL (35.9%), amoxicillin (20.5%), nitrofurantoin (17.9%), gentamycin HL (17.9%), ampicillin (7.7%) and vancomycin (0%).

**Table 5. Antimicrobial resistance profile among clinical enterococcal isolates (n=39)**

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>No. % of resistant enterococcal strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. faecalis</em> (n=32)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>2 6.3%</td>
</tr>
<tr>
<td>Gentamycin HL</td>
<td>6 18.9%</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0 -</td>
</tr>
<tr>
<td>Streptomycin HL</td>
<td>11 34.4%</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>5 15.6%</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>21 65.6%</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>17 53.1%</td>
</tr>
<tr>
<td>Amoxycilin</td>
<td>0 -</td>
</tr>
<tr>
<td>Amoxycilin-clavulanate</td>
<td>0 -</td>
</tr>
</tbody>
</table>

*NT- Not tested. Test for amoxicillin-clavulanate susceptibility only carried out on strains resistant to ampicillin.*

**4.2.2 Patient stools’ isolates.**

Enterococcal resistance was much lower among isolates obtained from patient stools compared to that obtained from clinical isolates. No isolate was resistant to ampicillin or vancomycin. Among the isolates obtained from patient stool only one was found to be resistant to nitrofurantoin.

*E. faecium* was more resistant than *E. faecalis* and other species to most antibiotics tested except for gentamycin where other enterococcal species showed a marginally higher resistance (*E. faecalis*=5.3%, *E. faecium*=12.5% and other species=13.3%).

There was no statistically significant difference in the rate of resistance between *E. faecalis* and that of *E. faecium* isolates in this category to the drugs tested namely gentamycin HL ($\chi^2$=0.024, $p>0.1$) streptomycin HL ($\chi^2$=0.47, $p=0.1$), ciprofloxacin ($\chi^2$=0.09, $p=0.1$), chloramphenicol
(χ²=0.065, p>0.1) and amoxicillin (χ²=0.043, p>0.1) in all cases. The test was not performed for ampicillin and vancomycin, since there was no resistance detected to them. Similarly, the test was not performed for nitrofurantoin since resistance was detected only to *E. faecium*. Resistance to amoxicillin-clavulanate was not tested since no isolate was found to be resistant to ampicillin.

*E. faecium* showed more resistance to streptomycin HL (*E. faecium* = 25%, *E. faecalis*=10.5%, *other species* =6.7%), to ciprofloxacin (*E. faecium* = 31.3%, *E. faecalis*=21%, *other species* =20%), to chloramphenicol (*E. faecium* = 25%, *E. faecalis*=15.8%, *other species* =20%), and to amoxicillin (*E. faecium*=18.8%, *E. faecalis*=10.5%, *other species*=6.7%). See table 5.

Overall resistance to antimicrobials in patient stools’ isolates was ciprofloxacin (24%), chloramphenicol (20%), streptomycin HL (14%), gentamycin HL (10%), amoxicillin (10%) and nitrofurantoin (2%). There was no resistance to ampicillin or to vancomycin.

**Table 6. Antimicrobial resistance profile among enterococcal isolates from patient stools (n=50)**

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>No. % of resistant enterococcal strains</th>
<th>E. faecalis (n=19)</th>
<th>E. faecium (n=16)</th>
<th>Other species (n=15)</th>
<th>Overall (n=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gentamycin HL</td>
<td></td>
<td>1</td>
<td>5.3%</td>
<td>2</td>
<td>12.5%</td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Streptomycin HL</td>
<td></td>
<td>2</td>
<td>10.5%</td>
<td>4</td>
<td>25%</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td></td>
<td>1</td>
<td>5.3%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td></td>
<td>4</td>
<td>21%</td>
<td>5</td>
<td>31.3%</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td></td>
<td>3</td>
<td>15.8%</td>
<td>4</td>
<td>25%</td>
</tr>
<tr>
<td>Amoxycilin</td>
<td></td>
<td>2</td>
<td>10.5%</td>
<td>3</td>
<td>18.8%</td>
</tr>
<tr>
<td>Amoxycilin-clavulanate</td>
<td></td>
<td>NT</td>
<td>-</td>
<td>NT</td>
<td>-</td>
</tr>
</tbody>
</table>

*NT- Not tested. Test for amoxicillin-clavulanate susceptibility only carried out on strains resistant to ampicillin.*

### 4.2.3 Healthy control stool isolates

There was very little resistance to antibiotics by enterococcal strains from healthy control’s stools (Table 7). There was no resistance to ampicillin, vancomycin and nitrofurantoin. The most common drug resistance was to ciprofloxacin (10%) and to chloramphenicol (8%). *E. faecium* exhibited the most resistance followed by other non-*faecium* non-*faecalis* enterococcal species. *E. faecalis* among controls had virtually no resistance only one strain to gentamycin and one strain to ciprofloxacin. Resistance to amoxicillin-clavulanate was not tested since no strain was
resistant to ampicillin. There was no statistically significant difference in the rate of resistance between \textit{E. faecalis} and that of \textit{E. faecium} isolates from healthy control stools to the drugs tested namely gentamycin HL ($\chi^2=0.013$, $p > 0.1$) and ciprofloxacin ($\chi^2=0.16$, $p > 0.1$). A comparison of \textit{E. faecalis} and \textit{E. faecium} resistance to ampicillin, vancomycin and nitrofurantoin was not possible since there was no drug resistance to these drugs by either \textit{E. faecalis} and/or \textit{E. faecium}. Similarly, no analysis comparing drug resistance to chloramphenicol and amoxicillin was possible since there was not resistance to these drugs by \textit{E. faecalis}. See table 7.

Table 7. Antimicrobial resistance profile among enterococcal isolates from healthy control’s stools (n=50)

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>No. % of enterococcal strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{E. faecalis} (n=15)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0 -</td>
</tr>
<tr>
<td>Gentamycin HL</td>
<td>1 6.7%</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0 -</td>
</tr>
<tr>
<td>Streptomycin HL</td>
<td>0 -</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>0 -</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1 6.7%</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0 0</td>
</tr>
<tr>
<td>Amoxycilin</td>
<td>0 0</td>
</tr>
<tr>
<td>Amoxycilin-clavulanate</td>
<td>NT -</td>
</tr>
</tbody>
</table>

4.2.4 Comparison of resistance of \textit{E. faecium} and \textit{E. faecalis} between clinical and non-clinical isolates.

\textit{E. faecalis}

Clinical isolates versus healthy control stool isolates

There was statistically significant difference in the frequency of resistance between clinical isolates and healthy control stool isolates of \textit{E. faecalis} to ciprofloxacin only ($\chi^2 = 11.99$, $p < 0.001$). No such difference was observed for gentamycin ($\chi^2 = 0.42$, $p > 0.1$). The test was not performed for the other drugs since all 15 \textit{E. faecalis} isolates from healthy control stool were susceptible to all the other drugs.
Clinical isolates versus patient stool isolates
There was statistically significant difference in the frequency of resistance between clinical isolates and patient stool isolates of *E. faecalis* to ciprofloxacin ($\chi^2 = 7.78$, $p < 0.01$) and chloramphenicol ($\chi^2 = 5.49$, $p < 0.025$). No such difference was observed for gentamycin ($\chi^2 = 0.87$, $p > 0.1$), streptomycin ($\chi^2 = 2.42$, $p > 0.1$), and nitrofurantoin ($\chi^2 = 0.44$, $p > 0.1$).

*E. faecium*

Clinical isolates versus healthy control stool isolates
There was no statistical difference in the frequency of resistance observed between *E. faecium* clinical and healthy control isolates. Gentamycin ($\chi^2 = 0.16$, $p > 0.1$), streptomycin ($\chi^2=1.89$, $p > 0.1$), ciprofloxacin ($\chi^2 = 1.02$, $p > 0.1$), chloramphenicol ($\chi^2 = 0.33$, $p > 0.1$). The test was not performed for the other drugs since resistance was zero in either of the groups.

Clinical isolates versus patient stool isolates
There was no statistical difference in the frequency of resistance observed between *E. faecium* clinical and patient stool isolates. Gentamycin ($\chi^2 = 0.20$, $p > 0.1$), streptomycin ($\chi^2=0.37$, $p > 0.1$), ciprofloxacin ($\chi^2 = 0.1$, $p > 0.1$), chloramphenicol ($\chi^2 = 0.021$, $p > 0.1$). The test was not performed for the other drugs since resistance was zero in either of the groups.

4.2.5 Comparison of overall resistance of the enterococci between clinical and non-clinical isolates.

Gentamycin
There was no significant difference in the frequency of drug resistance to gentamycin between clinical and non-clinical isolates ($\chi^2=0.60$, $p > 0.1$) and between patients’ stool isolates and healthy control stool isolates ($\chi^2=0.10$, $p > 0.1$).

Streptomycin
There was significant difference in the frequency of drug resistance to streptomycin between clinical and non-clinical isolates ($\chi^2=4.68$, $p <0.05$). There was however no statistical significance in the difference of resistance to streptomycin between patient stool and control stool isolates ($\chi^2=1.0$, $p > 0.1$).
**Nitrofurantoin**
The difference in resistance to nitrofurantoin between clinical and stool strains was statistically significant ($\chi^2=5.0, p > 0.05$).

**Ciprofloxacin**
There was significant difference in the frequency of drug resistance between clinical isolates and stool isolates to ciprofloxacin ($\chi^2=11.31, p > 0.005$). There was no significant difference in the frequency of drug resistance to ciprofloxacin between patient stool isolates and control stool isolates ($\chi^2=2.55, p > 0.1$).

**Chloramphenicol**
There was significant difference in the frequency of drug resistance to chloramphenicol between clinical isolates and stool isolates ($\chi^2=6.97, p > 0.01$). The observed difference in resistance to chloramphenicol between patient stool isolates and control stool isolates was not significant ($\chi^2=2.17, p > 0.1$).

**Table 8. Statistical difference in the frequency of drug resistance between clinical and non-clinical enterococcal isolates.**

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Statistically significant difference in the frequency of drug resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clinical vs non-clinical isolates</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>NO</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>YES</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>YES</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>YES</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>YES</td>
</tr>
</tbody>
</table>

*N/B: The drugs whose difference is not presented in this table (Ampicillin, Vancomycin and amoxycillin), returned a value of zero resistance in one of the categories under comparison. The Chi square could therefore not be calculated.*

*N/A, Nitrofurantoin similarly returned zero resistance to healthy control stool isolates.*
CHAPTER V - DISCUSSION

5.1 Distribution of enterococcal species

Out of the 139 samples from which enterococcus species were recovered, *E. faecalis* (82%) were the most prevalent followed by *E. faecium* (15.4%). Other enterococcal species which were neither *E. faecalis* nor *E. faecium* accounted for 2.6%. These findings were similar to those of other studies. According to Patel *et al* (2011), *E. faecalis* contributed 67% of 36 clinical enterococcal isolates, while *E. faecium* contributed 19%. The other enterococcal species were 14%. In a study conducted in Brazil (Ricardo *et al*. 2003), out of 99 clinical enterococcal isolates, *E. faecalis* accounted for 76%, *E. faecium* 9% and other enterococcal species accounted for 15%.

*E. faecalis* was the only species recovered from blood and ascitic fluid. Gray *et al* (1991) reported that most studies show that *E. faecalis* is the most predominant enterococcal blood culture isolate.

Each specimen provided only one isolate since only one pure colony was used in the purification step. There was therefore no overlap where a specimen could provide more than one enterococcal species.

Unlike the distribution in non-stool clinical isolates, *E. faecium* and other enterococcal species were more represented in stool. There was no significant difference in the distribution of enterococcus species in stool specimen of patients and healthy controls.

5.2 Antimicrobial susceptibility patterns

All enterococcal isolates in this study were sensitive to vancomycin. This is particularly important with respect to therapy of enterococcal infections since vancomycin is a last line drug for treatment of infections with these pathogens. Similar results were observed in other studies. Zouain and Araj (2001) reported that out of 153 clinical enterococcal isolates, none showed resistance to vancomycin. According to Shrishari *et al* (2011), among 54 clinical enterococcal samples tested, none showed resistance to vancomycin. However, some studies have reported either very low or sporadic resistance to vancomycin by clinical enterococcal isolates. Gordon *et
al (1992) reported that out of 705 clinical specimens, only two E. faecalis isolates were resistant to vancomycin. According to Anita et al. (2001), in a study involving 322 clinical enterococcal isolates, resistance to vancomycin was encountered only sporadically, with only two E. faecium isolates showing resistance.

In general E. faecium showed more drug resistance than E. faecalis isolates to ampicillin, streptomycin HL, nitrofurantoin and amoxicillin. This observation is in agreement with other findings (Shrishari et al., 2011; Huycke et al., 1998; Gray et al., 1991; Low et al., 2001; Anita et al., 2001). These studies show that E. faecium exhibits greater resistance compared to E. faecalis. According to Huycke et al. (1998) and Mundy et al. (2000), E. faecalis is more likely to express traits related to overt virulence but also more likely to retain sensitivity to at least one effective antibiotic, while E. faecium, is a species virtually devoid of known overt pathogenic traits but more likely to be resistant to even antibiotics of last resort. E. faecalis was however more resistant than E. faecium to ciprofloxacin and to chloramphenicol.

Complete or relative resistance to β-lactams such as ampicillin is a characteristic feature of the genus Enterococcus. According to Murray (1997), E. faecalis is typically 10 to 100 times less susceptible to penicillin than are most streptococci, while E. faecium is at least 4 to 16 times less susceptible than E. faecalis. The major mechanism underlying this resistance has been the production of low-affinity penicillin binding proteins (PBP). E. faecium are less susceptible to β-lactams than E. faecalis because the penicillin binding proteins of E. faecium have markedly lower affinity for these antibiotics (Huycke et al., 1998). While most isolates of E. faecalis are inhibited by concentrations of penicillin or ampicillin (1 to 8 µg/ml) easily achievable in humans, isolates of E. faecium usually require an average of 16 to 64 µg/ml to inhibit growth, although some isolates are even more resistant (Murray, 1997). Results from the SENTRY Antimicrobial Surveillance Program, 1997–1999 (Low et al., 2001) showed that 17% of enterococcal isolates from the United States of America were resistant to ampicillin, compared to 1% of strains from Latin America. E. faecium was most prevalent (20% of enterococcal isolates) in the United States, explaining the relatively higher resistance rates, compared to 4.6% prevalence in Latin America.

Resistance to aminoglycosides such as streptomycin is thought to be due to production of aminoglycoside-inactivating enzymes (Moellering and Weinberg, 1971). It is thus possible that
*E. faecium* may be endowed with greater enzyme production efficiency than *E. faecalis*. Additionally another mechanism of resistance to streptomycin is due to low level permeability which can be overcome by penicillin (Moellering and Weinberg, 1971). Since *E. faecium* has penicillin binding proteins with markedly lower affinity, its resistance to streptomycin can be more pronounced than that of *E. faecalis*. It is thus expected that susceptibility patterns though not to certain specific agents, should show *E. faecium* being more resistant than *E. faecalis* and other enterococcal species.

a) Clinical enterococcal isolates

Among clinical strains overall resistance to ampicillin was 7.7% and to high level gentamycin (HL) was 17.9 %, to streptomycin HL was 35.9%. These three drugs are first line in the treatment of severe enterococcal infections. Ampicillin is the drug of choice for treatment of enterococcal infections. Therefore, the resistance observed (7.7% overall, 6.3% for *E. faecalis* and 16.7% for *E. faecium* isolates) is significant especially from a clinical perspective. According to a study carried out in India (Patel *et al.*, 2011), the resistance of clinical enterococcal isolates to ampicillin and gentamycin was 30.6% and 55.5% respectively. In a study of enterococcal isolates from hospitalized patients in England (Gray *et al.*, 1991), 63.2% of *E. faecium* isolates were resistant to ampicillin, while ampicillin resistance was not found in *E. faecalis* isolates.

Results from the SENTRY Antimicrobial Surveillance Program, 1997–1999, (Low *et al.*, 2001) showed that 17% of enterococcal isolates from the United States of America were resistant to ampicillin. These reports emphasize the need for identification and susceptibility tests on all enterococcal clinical isolates in order to ensure the most appropriate approach to therapy of enterococcal infections.

There was however, no statistically significant difference in the rate of resistance between *E. faecalis* and that of *E. faecium* isolates in this category of clinical isolates to ampicillin, gentamycin, streptomycin, nitrofurantoin, ciprofloxacin, and chloramphenicol. The test was not performed for vancomycin since they all returned a value of zero in all the categories (resistances).
Resistance to ampicillin (7.7%) was observed among clinical isolates only. There was no such resistance among isolates from stool samples both from patients and from controls. Most literature on enterococci do not report comparative data between clinical isolates and healthy control stool isolates. There are therefore no reports to compare with these results. However, one possibility is that the clinical infections may have been caused by multidrug resistant strains that have been selected over time by antibiotic pressure especially in the hospital setting and thus have greater potential for resistance to a wider range of antibiotics and could also be nosocomially transmitted strains. Although the medical history of the patients was not recorded, the most likely situation would be that the strains resistant to the various antimicrobials were from patients hospitalized for significant lengths of time and may have acquired nosocomially transmitted enterococcal strains.

Among clinical isolates *E. faecalis* showed higher resistance than *E. faecium* to HL gentamycin, ciprofloxacin and chloramphenicol. This observation was not seen among non clinical samples. A correlation between high level gentamycin resistance and a likelihood of higher resistance to fluoroquinolones and chloramphenicol has previously been observed (Schouten et al., 1999). In that study, 79% of *E. faecalis* isolates with high level resistance (HLR) to gentamycin were resistant to ciprofloxacin, and only 52% of *E. faecium* with HLR to gentamycin were resistant to ciprofloxacin. This suggests a linkage between gentamycin resistance and resistance to fluoroquinolones. This linkage was however unexplained. Strains with HLR to gentamycin were also more likely to be resistant to chloramphenicol. Similar correlation was also observed by Vandamme *et al* (1996), in a study carried out in Belgium. That study found a high-level *E. faecalis* resistance to streptomycin (50.8%) and to gentamycin (8.7%).

Emergence of ciprofloxacin resistance was found to be associated with aminoglycoside resistance. Anita *et al* (2001) also showed a concomitant resistance to fluoroquinolones for *E. faecalis* isolates with HLR to gentamycin. The existence of such a linkage explains the higher resistance to ciprofloxacin among *E. faecalis* isolates as compared to *E. faecium* isolates.

b) Patient stool isolates

The major findings in respect to isolates from patient stools was that enterococci isolated from them had lower drug resistance compared to resistance among clinical isolates. There was no
statistically significant difference in resistance between *E. faecalis* and *E. faecium* isolates in this category to all the drugs tested. The enterococcal stool isolates from hospitalized patients may be normal enteric commensals that have been exposed to drug regimens during the patient treatment period. This exposure may have rendered the isolates slightly resistant to certain drugs but do not exhibit the characteristic transmissible resistance encoded by plasmids that may be responsible for MDR traits as observed in clinical isolates. It has been shown that even after a few days of hospitalization, strains representing the physiological flora of the patient are replaced by hospital strains highly resistant to antibiotics (Dzierzanowska, 1999).

In a study of susceptibility of enterococcal isolates from hospitalized patients in the UK, 63% of *E. faecium* isolates were resistant to ampicillin while all *E. faecalis* isolates were susceptible (Gray *et al.* 1991). However, high-level gentamycin resistance was found in 8.2% of *E. faecalis* isolates and not in other species. This observation supports the theory that hospitalization and drug exposure can result in acquisition of resistance by enterococcal strains colonizing the concerned patients or that the patients can easily acquire drug resistant strains from the hospital.

c) Control stool isolates

The major findings as regards healthy controls’ isolates in the present study were that they in general exhibited little resistance to all antibiotics, they showed highest resistance to ciprofloxacin and chloramphenicol and that *E. faecium* isolates showed highest resistance than *E. faecalis* and other species. The observation that *E. faecium* showed higher resistance among the healthy controls’ isolates can be explained from the fact that most studies have demonstrated that *E. faecium* is the most resistant enterococcus by natural intrinsic factors (Huycke *et al.*, 1998). These healthy controls had no recent exposure to drugs and had not been hospitalized within the last one month. Commensal enterococci from such individuals will thus be expected to exhibit only the intrinsic resistance of enterococci without any enhancement.

This intrinsic resistance may explain the observed trend that resistance to ciprofloxacin and chloramphenicol were highest in all three categories of isolates, clinical, patient stools and healthy controls (compared to other drugs). Similarly, in a study of enterococci intrinsically resistant to vancomycin (Possessing the vanC Genotype), the rate of VANC VRE recovery from the stools of patients and that of controls from the community was not significantly different.
(χ²=0.00089, p > 0.1) (Toye et al., 1997). This implies a shared property of intrinsic nature. This observation supports the idea that controls are colonized by enterococci that exhibit only intrinsic resistance and are devoid of MDR traits. This might therefore be the reason for lower resistance seen in isolates from healthy controls compared to hospitalized patients.

d) All categories of isolates

Resistance to ciprofloxacin and chloramphenicol was higher in all three categories of isolates clinical, patient stools and healthy controls. The reason for this observation could be intrinsic factors that are also unique to isolates in this part of the world. In a study designed to monitor the spectrum of microbial pathogens concurrent with antimicrobial resistance trends for both nosocomial and community-acquired infections on a global scale (Low et al., 2001), continental variations in antimicrobial resistance were established. It is thus possible to find variations in trends of drug resistance from one part of the world to another.

The observed overall patterns of resistance might also be contributed by the procedure used to measure the level of resistance. In a study of enterococci susceptibility comparing six standard disk diffusion procedures, variations in the resistance patterns were associated with ciprofloxacin, gentamycin and nitrofurantoin (Cotter and Adley, 2001). This study showed that there may be variations in susceptibility patterns where these drugs are concerned and may explain some observed patterns in the present study especially with regard to ciprofloxacin.

There was no statistically significant difference in resistance between patient stool isolates and healthy control stool isolates for gentamycin HL, streptomycin HL, ciprofloxacin, and chloramphenicol. This means that there is no difference in resistance among enterococcal strains colonizing healthy persons and hospitalized patients free from MDR strains. In the study by Toye et al. (1997), where the recovery of van C VRE was similar in patients and healthy controls, none of the controls were positive for a VRE of either the van-A or the van-B genotype. Both van-A and van-B are found on a transposon or plasmid and thus transferable by conjugation. This further supports the theory that enterococci strains causing infections are those that have acquired drug resistance traits and have thus enhanced their existence and spread (Huycke et al., 1998).
There was statistically significant difference in resistance between clinical and non-clinical strains to streptomycin, nitrofurantoin, ciprofloxacin, and chloramphenicol. This observation indicates that strains causing clinical infections exhibit enhanced drug resistance compared to enterococci that are not associated with clinical infection. These isolates causing clinical infections are strains that exhibit multiple drug resistance traits and thus selected by antibiotic pressure and are more likely to cause infection than other strains and also be transmitted nosocomially. Only gentamycin did not show statistically significant difference in resistance between clinical and non-clinical isolates. This could mean that the drug has better efficacy against all forms of enterococci. These MDR strains have not acquired resistance to gentamycin.
CHAPTER VI – CONCLUSIONS & RECOMMENDATIONS

6.1 Conclusions

All isolates in this study were sensitive to vancomycin. This implies that vancomycin is still effective as a last line drug for treatment of severe enterococcal infections.

*E. faecium* showed more resistance than *E. faecalis* isolates in general. This observation is in agreement with most literature.

Among clinical strains overall drug resistance was 7.7% to ampicillin, 17.9% to gentamycin HL and 35.9% to streptomycin HL. These three drugs are first line in the treatment of severe enterococcal infections. The observed results and similar reports from other studies emphasize the need for identification and susceptibility tests on all enterococcal clinical isolates in order to ensure the most appropriate approach to therapy of enterococcal infections.

There was no resistance to ampicillin among enterococci isolated from non-clinical samples and there was no statistical significance to the difference in resistance observed for gentamycin between clinical and non-clinical isolates as well as between patient stool isolates and control stool isolates. This shows that ampicillin and gentamycin are effective against enterococci.

The rate of resistance observed for streptomycin is statistically significant and raises concern regarding the future usefulness of this drug in therapy of enterococcal infections.

Statistical analysis of drug resistance indicate that strains causing clinical infections exhibit enhanced drug resistance compared to enterococci that are not associated with clinical infection. These isolates causing clinical infections are strains that exhibit multiple drug resistance traits and thus selected by antibiotic pressure and are more likely to cause infection than other strains and also be transmitted nosocomially.
6.2 Recommendations

The clinical microbiology laboratory plays an important role in the detection, timely reporting, and control of multi-drug resistant enterococci.

In order to effectively monitor the development of resistance to antimicrobials by enterococci, and to ensure proper management of such infections, two elements are important to establish. First, it is essential to ensure that enterococci are rapidly and accurately identified to the species level in order to associate resistance to specific species and secondly, accurate testing of susceptibility should be performed to ensure appropriate use of antimicrobials in therapy of enterococcal infections.

Vancomycin has been shown to be effective against all enterococcal isolates. It is therefore recommended as last line drug for treatment of severe enterococcal infections.

Due to the effectiveness observed for ampicillin and gentamycin, the continued use of these two drugs as first line for the treatment of enterococcal infections is recommended.

Subsequent to the significant resistances observed, the use of nitrofurantoin, chloramphenicol, ciprofloxacin, and streptomycin for treatment of enterococcal infections should be subject to results of susceptibility testing.

Multiple drug resistant isolates have potential for infection and spread. It is therefore important to emphasize the importance of controlling the spread of enterococcal resistance. Educational programs for health care workers are thus needed to improve the use of antimicrobials.

Antibiotic therapy should avoid the use of glycopeptides (e.g. vancomycin) and newer drugs in order to reduce the drug selective pressure for resistant strains. This study shows that there is possible acquisition of resistance by clinical enterococcal isolates. There is therefore need for the rational use of antimicrobials as means of minimizing enterococcal infections. It is particularly important to perform susceptibility testing on all enterococcal clinical isolates in order to ensure the application of the most appropriate antibiotics for treatment.
APPENDIX I

ANTIBIOTIC DISK SUSCEPTIBILITIES
(KIRBY-BAUER DISK-DIFFUSION METHOD)

When a 6-mm filter paper disk impregnated with a known concentration of an antimicrobial compound is placed on a Mueller-Hinton (MH) agar plate, immediately water is absorbed into the disk from the agar. The antimicrobial begins to diffuse into the surrounding agar. The rate of diffusion through the agar is not as rapid as the rate of extraction of the antimicrobial out of the disk, therefore the concentration of antimicrobial is highest closest to the disk and a logarithmic reduction in concentration occurs as the distance from the disk increases. The rate of diffusion of the antimicrobial through the agar is dependent on the diffusion and solubility properties of the drug in MH agar and the molecular weight of the antimicrobial compound. Larger molecules will diffuse at a slower rate than lower molecular weight compounds. These factors, in combination, result in each antimicrobial having a unique breakpoint zone size indicating susceptibility to that antimicrobial compound.

If the agar plate has been inoculated with a suspension of the pathogen to be tested prior to the placing of disks on the agar surface, simultaneous growth of the bacteria and diffusion of the antimicrobial compounds occurs. Growth occurs in the presence of an antimicrobial compound when the bacteria reach a critical mass and can overpower the inhibitory effects of the antimicrobial compound. The estimated time of a bacterial suspension to reach critical mass is 4 to 10 hours for most commonly recovered pathogens, but is characteristic of each species, and influenced by the media and incubation temperature. The size of the zone of inhibition of growth is influenced by the depth of the agar, since the antimicrobial diffuses in three dimensions, thus a shallow layer of agar will produce a larger zone of inhibition than a deeper layer.

The point at which critical mass is reached is demonstrated by a sharply margined circle of bacterial growth around the disk. The concentration of antimicrobial compound at this margin is called the critical concentration and is approximately equal to the minimum inhibitory concentration obtained in broth dilution susceptibility tests.
Zone size observed in a disk diffusion test has no meaning in and of itself (7). The interpretation of resistance and susceptibility to antimicrobials is determined through in vivo testing of blood and urine to calculate the obtainable level of a given antimicrobial that results in resolution of an infection. This information is correlated with zone sizes resulting in the interpretive standards.

Procedure:

1. Make a suspension at an appropriate turbidity of the bacterial culture to be tested.

2. Place a sterile cotton swab in the bacterial suspension and remove the excess fluid by pressing and rotating the cotton against the inside of the tube above the fluid level. The swab is streaked in at least three directions over the surface of the Mueller-Hinton agar to obtain uniform growth. A final sweep is made around the rim of the agar. Be sure to streak for confluency.

3. Allow the plates to dry for five minutes.

4. Using sterile forceps, place disks containing the appropriate antibiotics on the plate: e.g. penicillin G, ampicillin, streptomycin.

5. Incubate the plates within 15 minutes after applying the disks. The plates should be incubated soon after placing the disks since the test is standardized under conditions where diffusion of the antibiotic and bacterial growth commence at approximately the same time.

6. Following overnight incubation, measure the diameter of the zone of growth inhibition around each disk to the nearest whole mm. Examine the plates carefully for well-developed colonies within the zone of inhibition.

7. Using a standard table of antibiotic susceptibilities, determine if the strain is resistant, intermediate, or susceptible to the antibiotics tested.
APPENDIX II

API 20 STREP SYSTEM

API 20 STREP is a standardized method consisting of 20 biochemical tests that offer widespread capabilities. It enables group or species identification of most streptococci encountered in medical bacteriology. It consists of a strip of 20 microtubules containing dehydrated substrates for the demonstration of enzymatic activity or the fermentation of sugars.

The enzymatic tests are inoculated with a dense suspension of organisms, made from a pure culture, which is used to rehydrate the enzymatic substrates. The metabolic end products produced during the incubation period are either revealed through spontaneous colored reactions or by the addition of reagents.

The fermentation tests are inoculated with an enriched medium, which reconstitutes the sugar substrates. Fermentation of carbohydrates is detected by a shift in the pH indicator. The reactions are read according to the reading table and the identification is obtained by referring to the analytical profile index or using the identification software.

The API 20 STREP system is intended uniquely for the identification of Streptococcus, Enterococcus, Listeria, Gardenella, and Aerococcus species included in the database. It cannot be used to identify any other microorganisms or to exclude their presence.
APPENDIX III
CONSENT FORM FOR SPECIMEN COLLECTION

Principal investigator
Dr. Gideon Nkeyasha, Department of Medical Microbiology, University of Nairobi
Tel. 0722894268.

Introduction
I am conducting a study on patterns of drug resistance to a bacterium that is commonly found in the intestinal tract of humans including patients and healthy persons, and will be grateful if you could agree to participate in it. The study will involve examining specimen obtained from willing patients and college students, if it has this bacterium and if the bacterium is resistant to several antibiotics.

Purpose of the research
This study is aimed at finding out if a bacteria known as enterococci found in specimen obtained from patients and controls is resistant to certain antibiotics, and if the pattern of this resistance differs between the two groups. This information will be useful in deciding on how to treat infection with this bacterium, which is a common cause of infections acquired in and around hospitals.

Procedures
Stool or other specimen obtained from you will be examined in the laboratory and tested for antibiotic resistance using specialized techniques. The results obtained from patients and controls will be compared.

Participation
Participation in this study is completely voluntary. Information on the study is provided in this consent form. Once you understand what it takes and you are willing to participate, you will be asked to indicate this by signing and dating the attached form. A copy of the signed and dated form will be given to you to keep if you are interested.
Confidentiality
All information obtained in this study will be kept highly private and confidential

Benefits

You may not personally benefit from the results of this study. However the information obtained may be communicated to hospital staff concerned with the management of infections, to help improve treatment of infections with this bacterium.

Questions

If you have any questions about the study contact Dr. Gideon Nkeyasha on 0722894268 or gnkeyasha@yahoo.com or gnkeyasha09@gmail.com.

Queries

In case of any queries regarding this project, contact the chairman of the Kenyatta National Hospital and University of Nairobi ethics and research committee as given below.

Prof. K.M. Bhatt, Chairperson, KNH/UON-ERC
P.O. Box 20723, Nairobi
Tel: 726300-9
Fax: 725272

Statement of consent

I have read this form or had it read to me. I have discussed the information with those concerned. All my concerns have been answered. I understand that my decision to take part in the study is voluntary. By signing this form I do not give up any rights that I have as a research participant.

Participant’s name: _____________________________  Participant’s signature: ____________
________________________________________________________________________

Date: _____________________________

Principal investigator

Dr. Gideon O. Nkeyasha  Signature _____________________________

Date _____________________________
APPENDIX IV

QUESTIONNAIRE FOR HEALTHY CONTROLS
(Kenya Polytechnic University college students)

(TICK AS APPROPRIATE)

1. Are you currently under any medical examination?
   □ Yes. □ No.

2. Are you currently taking any antibiotics?
   □ Yes. □ No.

3. Have you taken any antibiotics within the last one month?
   □ Yes. □ No.

4. Have you been hospitalized within the last one month?
   □ Yes. □ No.
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